Recommended Laboratory Methods for Assessment of Fish Quality

A.D. Woyewoda[1], S.J. Shaw[1], P.J. Ke and B.G. Burns

Department of Fisheries and Oceans
P.O. Box 550
Halifax, Nova Scotia, B3J 2S7

[1]Technical University of Nova Scotia

August 1986

Canadian Technical Report of Fisheries and Aquatic Sciences No. 1448

86-05570/

Canadian Technical Report of Fisheries and Aquatic Sciences

Technical reports contain scientific and technical information that contributes to existing knowledge but which is not normally appropriate for primary literature. Technical reports are directed primarily toward a worldwide audience and have an international distribution. No restriction is placed on subject matter and the series reflects the broad interests and policies of the Department of Fisheries and Oceans, namely, fisheries and aquatic sciences.

Technical reports may be cited as full publications. The correct citation appears above the abstract of each report. Each report is abstracted in *Aquatic Sciences and Fisheries Abstracts* and indexed in the Department's annual index to scientific and technical publications.

Numbers 1-456 in this series were issued as Technical Reports of the Fisheries Research Board of Canada. Numbers 457-714 were issued as Department of the Environment, Fisheries and Marine Service, Research and Development Directorate Technical Reports. Numbers 715-924 were issued as Department of Fisheries and the Environment, Fisheries and Marine Service Technical Reports. The current series name was changed with report number 925.

Technical reports are produced regionally but are numbered nationally. Requests for individual reports will be filled by the issuing establishment listed on the front cover and title page. Out-of-stock reports will be supplied for a fee by commercial agents.

Rapport technique canadien des sciences halieutiques et aquatiques

Les rapports techniques contiennent des renseignements scientifiques et techniques qui constituent une contribution aux connaissances actuelles, mais qui ne sont pas normalement appropriés pour la publication dans un journal scientifique. Les rapports techniques sont destinés essentiellement à un public international et ils sont distribués à cet échelon. Il n'y a aucune restriction quant au sujet; de fait, la série reflète la vaste gamme des intérêts et des politiques du ministère des Pêches et des Océans, c'est-à-dire les sciences halieutiques et aquatiques.

Les rapports techniques peuvent être cités comme des publications complètes. Le titre exact paraît au-dessus du résumé de chaque rapport. Les rapports techniques sont résumés dans la revue *Résumés des sciences aquatiques et halieutiques*, et ils sont classés dans l'index annual des publications scientifiques et techniques du Ministère.

Les numéros 1 à 456 de cette série ont été publiés à titre de rapports techniques de l'Office des recherches sur les pêcheries du Canada. Les numéros 457 à 714 sont parus à titre de rapports techniques de la Direction générale de la recherche et du développement, Service des pêches et de la mer, ministère de l'Environnement. Les numéros 715 à 924 ont été publiés à titre de rapports techniques du Service des pêches et de la mer, ministère des Pêches et de l'Environnement. Le nom actuel de la série a été établi lors de la parution du numéro 925.

Les rapports techniques sont produits à l'échelon régional, mais numérotés à l'échelon national. Les demandes de rapports seront satisfaites par l'établissement auteur dont le nom figure sur la couverture et la page du titre. Les rapports épuisés seront fournis contre rétribution par des agents commerciaux.

CANADIAN TECHNICAL REPORT OF

FISHERIES AND AQUATIC SCIENCES NO. 1448

AUGUST 1986

RECOMMENDED LABORATORY METHODS FOR ASSESSMENT OF FISH QUALITY

BY

A.D. WOYEWODA[1], S.J. SHAW[1], P.J. KE[2] AND B.G. BURNS[2]

[1]Canadian Institute of Fisheries Technology
P.O. Box 1000
Halifax, Nova Scotia
B3J 2S7

[2]Department of Fisheries and Oceans*
P.O. Box 550
Halifax, N.S.
B3J 2S7

* Reprints

Minister of Supply and Services Canada 1986

Cat. No. Fs 97-4/1551 ISSN 0706-6473

ABSTRACT

Woyewoda, A.D., S.J. Shaw, P.J. Ke and B.G. Burns. 1986.
Recommended laboratory methods for assessment of fish quality.
Canadian Technical Report of Fisheries and Aquatic Sciences
No. 1448: V + 156 p.

The objective of this work was to provide standard techniques and procedures for laboratory grading of fish qualtiy to reduce disparity among testing facilities and provide a base from which technical staff and new personnel can obtain standard information.

The twenty-one laboratory procedures for scientific evaluation of fish quality have been reviewed and selected from the literature and in most cases, tested for applicability to Atlantic species. Each method incorporates an introductory section followed by application, principle of the method, precautions and notes, sample preparation, equipment, reagents, detailed procedure, calculations and typical examples.

Findings of laboratory testing undertaken during the compilation of this guide have been incorporated into the "procedures" and "precautions" sections. The methods are not "state-of-the-art" or mechanized but rather utilize long-standing procedures which may be performed in moderately equipped laboratories. In many cases, newer methods are mentioned. The recommended methods have been tested and used by four DFO regional inspection laboratories and some fisheries institutes in Canada, Norway, Japan and China. The comments concerning application of these techniques to various quality assessment operations in both research and routine work have been positive.

RÉSUMÉ

Woyewoda, A.D., S.J. Shaw, P.J. Ke and B.G. Burns. 1986.
Recommended laboratory methods for assessment of fish quality.
Canadian Technical Report of Fisheries and Aquatic Sciences
No. 1448: V + 156 p.

L'objectif de ce travail était de pourvoir les laboratoires d'analyses de techniques et procédés standards pour la classification de la qualité du poisson, de procurer aux chercheurs, techniciens et nouveaux employés les informations necessaires à cet effet et de réduire la disparité parmis ces établissements.

Les 21 analyses d'évaluation scientifique de la qualité du poisson, tirées de la littérature éxistante, ont été revisées et pour la plupart essayées avec des espèces de poisson de l'atlantique. Chaque méthode est composée d'un préambule suivi de l'application du principe de la méthode, des précautions à prendre, de la préparation des échantillons, d'une description de l'équipement, des réactifs, de la procédure detaillées, des calculs et d'exemples typiques.

Les résultats d'analyses entrepris pendant la compilation de ce guide ont été incorporés dans les sections "procédés" et "précautions". Ses méthodes ne sont pas parfaites ou mécanisées mais elles utilisent plutôt des procédés anciens, lesquels peuvent être exécutés dans un laboratoire modérément équipé. Dans plusieurs cas, des méthodes plus nouvelles sont mentionnées. Les méthodes recommendées ont été essayées et utilisées dans quatre laboratoires d'inspection régionals de Pêches et Océans et dans quelques instituts de recherche en pêcherie au Canada, en Norvège, au Japon et en Chine. Ses commentaires sont positifs quant à l'application de ces techniques dans différentes opérations d'évaluation de qualité tant pour la recherche que le travail de routine.

TABLE OF CONTENTS

ABSTRACT..... ii
RESUME..... iii
TABLE OF CONTENTS..... iv/v

INTRODUCTION..... 1

A. PROXIMATE COMPOSITION
1. Measurement of pH..... 2
2. Moisture Determination..... 6
3. Lipid Content (fat)..... 9
A. Fat Content Only..... 10
B. Fat Content With Recovery For Subsequent Analysis..... 11
4. Protein - Micro-Kjeldahl Analysis..... 15
5. Non-protein nitrogen..... 21
6. Ash content..... 26
7. Salt content..... 28
8. Bone content..... 32

B. QUALITY INDICES - REFRIGERATED AND ICED STORAGE
9. Trimethylamine (TMA)..... 35
10. Total Volatile Base (TVB)..... 41
A. Total Volatile Base (TVB-1) - Direct MgO Method..... 43
B. Total Volatile Base (TVB-2) -$MgSO_4$ Extraction Method..... 46
11. A. Hypoxanthine (Hx) (Enzymatic Method)..... 50
B. Hypoxanthine (Hx) (HPLC Method)..... 53
12. Simultaneous Dimethylamine (DMA)/ Trimethylamine (TMA) Determination..... 58

C. QUALITY INDICES - LIPID RELATED
13. Thiobarbituric Acid Test (TBA/TBARS)..... 65
A. Thiobarbituric Acid Reactive Substances (TBARS)..... 68
B. Tarladgis Thiobarbituric Acid Value (TBA)..... 69
14. Peroxide Value (POV)..... 73
15. Carbonyl Value (COV)..... 77
16. Free Fatty Acid (FFA)..... 82
A. For Tissue..... 84
B. For Lipid..... 85

D. QUALITY INDICES - NON-LIPID RELATED
17. Extractable Protein Nitrogen (EPN)..... 89
18. Dimethylamine (DMA)..... 96
19. Formaldehyde (FA)..... 101
20. G/P Value..... 106

E. PHYSICAL ATTRIBUTES
21. Color Measurement..... 112

TABLE OF CONTENTS

APPENDIX A. Development of Fish Grading Standards by Scientific Quality Assessment..... 117

APPENDIX B. Guide for Grading of Groundfish..... 122

APPENDIX C. Aids to Physical Grading..... 135

- INTRODUCTION -

Fish and seafood products are relatively unstable in fresh and frozen storage. Flavour and texture changes occur readily for a variety of reasons. In fresh (iced) storage, biochemical (i.e. enzymatic) and bacterial action produce specific measurable changes; in frozen storage, physical and enzymatic processes decrease quality.

Accurate measurement of physical character (composition) and "state-of-freshness" are important from both economic and processing efficiency aspects. To perform these assessments accurately and with confidence, and to be able to compare results between testing facilities, it is necessary that standard procedures be used. This collection of twenty-one laboratory methods is an attempt to compile such a standard. The procedures have been drawn from current literature and experience and have been tested by the Canadian Institute of Fisheries Technology or Fisheries and Oceans for their validity or appropriateness. Results of such testing were considered in the outlining of methodology.

The procedures have been divided into sections of proximate composition, quality indices of refrigerated or fresh storage, quality indices of frozen storage (lipid and non-lipid related) and physical attributes. Each method is followed by applicable references where additional information may be obtained regarding any aspect of the procedure, its application or interpretation.

1. MEASUREMENT OF pH

GENERAL DISCUSSION

The acidity of a substance is dependent on the number of hydrogen ions (H^+) which are ionized or dissociated when that substance is dissolved in water. Fresh milk for example has a very low hydrogen ion activity or concentration, approximately 2×10^{-7} or 0.0000002 ions per litre (1). In an aqueous solution an equilibrium exists between acidic (H^+) ions and basic (OH^-) ions (2) such that the mathematical product of H^+ and OH^- ion concentrations is constant, $K_w = 1 \times 10^{-14}$. Therefore in milk there are 2×10^{-7} H^+ and $10^{14} \div 2 \times 10^{-7} = 0.5 \times 10^{-7}$ OH^- ions per litre. Since H^+ concentration is slightly higher than OH^- concentraton, milk is although close to neutral, very slightly acidic. Hydrogen ion is more conveniently expressed as pH defined as the negative log of hydrogen ion concentration, i.e.

$$pH = -\log [H^+].$$

Therefore a solution with hydrogen ion concentration at 0.01 mole per litre has a pH of 2. Similarly, the pH of our milk sample is $-\log 2 \times 10^{-7} = 6.70$. Substances of pH lower than 7 are termed acidic and those higher than 7 basic. The pH of distilled water is usually below 7 since it absorbs carbon dioxide from the air which produces the unstable acid, carbonic acid (3). From the example it should be noted that at any pH, even in the extremes, there are always both H^+ and OH^- ions present although the concentration of any one may be very low. Since pH is a log function, a solution at any pH contains ten times as many hydrogen ions as an equal volume of a solution of the next higher pH (3).

The pH of living fish although generally between 6.7 and 7.0 is subject to variation with time of the year, feeding, and degree of activity (4). After death, glycogen stored in the muscle is broken down by glycolysis to produce lactic acid and in turn pH is lowered. This reduction accompanies the onset of rigor mortis where the fish muscle stiffens, muscle filaments shorten temporarily and in some cases of extreme pH depression, tearing of muscle tissue occurs to produce gaping fillets (5, 6). The time span over which these events take place and the resulting minimum pH achieved depend on the store of glycogen present in the fish muscle at the time of death along with a number of other factors including severety of handling and storage temperature.

In late winter and spring with the depletion of the food supply coupled with increased energy requirements for sex organ maturation, some body reserves are broken down resulting in a higher water content and a low store of glycogen (4). After death, the pH of such stored specimens is depressed only slightly causing a shortened ice shelflife. Immature fish which do not spawn are not subject to this effect. After spawning the fish begin to feed heavily and glycogen rises to very high levels. In this case, during rigor pH falls rapidly to low extremes, often below 6.0 (5, 7) inducing severe muscle shortening. Low pH fish store better on ice but toughen prematurely in frozen storage (4). Fortunately after a brief period of heavy feeding, the fish become more selective in their food and glycogen levels return to normal.

Once rigor has passed, bacterial activity produces a gradual increase of pH through the production of ammonia and other bases (8, 9). Therefore pH first decreases with the onset of rigor but then progressively increases from microbiological activity. For most fish, a pH value greater than 7.0 indicates spoilage (10).

Measurement of pH in fish muscle is best accomplished with a pH meter equipped with a glass combination pH electrode either directly on a mince or with distilled water added (10). The pH must be measured immediately after maceration or mixing with water (11) and caution must be exercised regarding temperature of sample and meter standardization. While some laboratories prefer blending fish muscle with 0.005 M sodium iodoacetate (11, 12) this method tends to yield pH values 0.16 unit lower on the average. Therefore direct or a water blending is recommended (10).

1. MEASUREMENT OF pH

Glass electrodes which are constructed of soft glass to display the necessary sensitivity toward the hydrogen ion exhibit "alkaline" and "acid" errors at extreme pH values.

At high pH, at 12 for example, the observed pH is lower than the true value because cations, particularly sodium, potassium and lithium compete for the exchange sites in the hydrated layers of the glass membrane and alter the phase-boundary potential (2). Similarly at low pH, near 0, observed pH is higher than the true value. For flesh pH measurements these phenomena are not important. However, since protein may coat the membrane and affect readings, proper care must be taken in cleaning the electrode, particularly after extended use. In some cases electrodes may need reconditioning (13).

a. Application

The measurement of pH is applicable to all fish and fish products.

b. Principle

The pH of a substance is measured after maceration with water (Method A) or directly (Method B) using a glass membrane electrode sensitive to hydrogen ions in combination with a saturated calomel reference electrode. The system provides a galvanic cell with phase boundaries across which measurable electromotive forces or potentials develop (2). The two electrodes for convenience are combined into a single "combination" electrode.

c. Precautions

1. The pH meter must be allowed sufficient time for warm-up.

2. The electrode must be kept clean and free of occluded protein. A weak base or a mild detergent solution may be used to clean the electrode. Soaking in 8 M urea for 2 hours may also help. At no time should abrasion be used since this will lead to permanent damage.

3. The pH meter must be standardized carefully 1 pH unit on either side of the anticipated pH to ensure linearity in the pH range being measured. Commercially available pH standard solutions are available for this purpose.

4. All standardization solutions and samples should be at room temperature (or at a common temperature) when being measured. The effect of temperature variation on pH of standards is noted on their label.

5. When drying the pH electrode, the tip should be patted with tissue rather than wiped to prevent the build up of static electricity in the electrode.

6. Use good quality fresh pH standards. Some standards change pH with age or on extended exposure to air.

7. Electrode should be stored immersed in distilled water between readings.

8. When the electrode is removed from a solution the meter should be placed on "standby".

9. All electrodes age resulting in a slower speed of response. Reconditioning may help restore electrode response. Recondition by immersing electrode tip 15 seconds in 0.1 N HCl rinsing in tap water, immersing for 15 seconds in 0.1N NaOH, rinsing and repeating each step several times. If this does not restore electrode, immerse in a 20% solution of NH_4 F.HF (ammonium bifluoride) for 2 to 3 minutes, rinse and check.

10. Some magnetic stirrers may influence readings. Check during standardization. It is not necessary to use a stirrer for routine pH checks of homogenous sample.

11. Several samples (specimens) should be used to compensate for biological variation among samples.

12. Sample pH must be determined immediately after maceration or blending of sample.

13. Samples should not be allowed to remain at room temperature for extended periods since bacterial activity will raise pH.

14. If using method B (without water addition) ensure good flesh/electrode contact.

SAMPLE PREPARATION

Representative samples should be rendered homogeneous by comminuting in a Cuisinart food processor and then immediately analyzed. Several samples should be used.

APPARATUS

1. **pH meter** equipped with combination pH electrode

2. **Blender** or food processor

3. **Glassware:** 25 ml and 50 ml beakers

4. **Thermometer**

REAGENTS

1. **Standard Buffers** ,commercial buffers 1 pH unit on either side of anticipated pH, usually pH 6 and 8 for flesh

PROCEDURE

Either method A (first choice) or method B may be applied.

METHOD A - with water addition

1. After sufficient warm-up time of the pH meter (ca 1/2 hr) set the "temperature" dial to 25°C.

2. Using pH 6 and pH 8 buffers in 25 ml beakers, standardize the meter to these values with the "buffer" and "temperature" adjustments. Depending on model of the meter, procedures for standardization may vary slightly. The instruction manual should be consulted. To take a reading vigorously stir the electrode in the sample and allow the electrode to rest against the beaker's wall. Allow time for the reading to stabilize. Rinse electrode with distilled water and pat dry with tissue between readings.

3. Repeat the standardization (step 2) until the meter provides accurate readings. A final check may be made with pH 7 buffer. Failure to achieve standardization may indicate electrode failure; recondition or replace.

4. Blend 20 g comminuted fish with 40 ml distilled water (at room temperature) for 1 minute in a blender.

5. Pour some of the slurry into a 50 ml beaker. Check temperature to ensure its proximity to that of the buffers.

6. Immediately insert rinsed and patted dry electrode. Stir the slurry vigorously with the electrode and allow the electrode to rest against the beaker's wall. After stabilization record the reading.

7. Place meter on "standby" and rinse electrode with distilled water.

8. Between readings store electrode in distilled water.

METHOD B - without water addition

1. Follow method A but in step 4 omit water addition using comminuted sample directly. Good electrode/flesh contact must be ensured.

REFERENCES

1. PAUL, C.P. 1972. Basic scientific principles, sugars and browning reactions. In Food Theory and Applications (Paul, C.P. and H.N. Palmer, editors) John Wiley & Sons, Inc., Toronto, pp 1-76.

2. FISCHER, R.B. and D.G. Peters. 1968. In Basic Theory and Practice of Quantitative Chemical Analysis (Fischer, R.B. and D.G. Peters, editors) W.B. Saunders Companay, Toronto, p. 251, 711.

3. CAMPBELL, A.M., M.P. Penfield, and R.M. Griswold. 1979. Introduction to food science. In The Experimental Study of Food. (Campbell, A.M., M.P. Penfield and R.M. Griswold, editors) Houghton Mifflin Company, Boston, pp 17-43.

4. LOVE, R.M. 1976. Processing cod: The influence of season and fishing ground. Torry Advisory Note No. 71, Torry Research Station, Aberdeen, Scotland.

5. PARTMANN, W. 1965. Changes in Proteins, nucleotides and carbohydrates during rigor mortis. In The Technology of Fish Utilization (R. Kreuzer, ed.) Fishing News (Books) Ltd. London.

6. STROUD, G.D. 1969. Rigor in fish. The effect on quality. Torry Advisory Note No. 36. Torry Research Station, Aberdeen, Scotland.

7. BRAMONAES, F., and P. Hansen. 1965. Technological problems connected with rigor mortis in fish requiring more knowledge from fundamental research. In The Technology of Fish Utilization (R. Kreuzer, ed.) Fishing News (Books) Ltd., London.

8. ESKIN, N.A., H.M. Henderson, and R.J. Townsen, 1971. Biochemistry of Foods. Academic Press, New York.

9. JACOBER, L.F. and A.G. Rand, Jr. 1982. Biochemical Evaluation of seafood. In Chemistry and Biochemistry of Marine Food Products (eds. R.E. Martin, G.J. Flick, C.E. Hebard and D.R. Ward) AVI Pub., Conn. pp 347-365.

10. VYNCKE, W. 1981. PH of fish muscle: Comparison of methods. Western European Fish Technologists Association, Copenhagen, 1981.

11. LIMA DOS SANTOS, C.A.M., D. James, and F. Teutscher. 1981. Guidelines for chilled fish storage experiments. FAO Fisheries Technical Paper No. 210. FAO, Rome.

12. MARCH, B. 1952. Biochem. Biophys. Acta. 9:127.

13. FISHER, J. E. Measurement of pH. 1984. American Laboratory 16 (6) 54.

2. MOISTURE DETERMINATION

GENERAL DISCUSSION

Water content or moisture affects food stability, inherent quality, processing potential and retail value (1, 2). An intimate functional relationship exists between water and other component substances such as proteins, fat and glycogen in living tissues (3); this balance plays a crucial role in the biochemistry of all living organisms.

The moisture content of fish varies with season and reproductive cycle. After spawning, fish tissue (groundfish in particular) is abnormally high in water, two to three percent higher than average. Flesh from such fish, generally of low pH appears wet, opaque and watery and is unsuitable for freezing since severe gaping will result (4). Fortunately this condition is present only for a very short time in the spring season.

In fatty fish, fat and water content have been correlated; often one measurement may be used for estimation of the other (5). Typical moisture values for most marine species are readily available in the literature (6, 7, 8).

A variety of moisture determination procedures are available and standard methods have been developed for most foods (1, 9, 10). Although newer technologies are being applied to moisture measurement (microwave, [11] etc.), the most widely used methods involve thermal drying under conditions which provide minimum loss of other volatile components. No method is perfect (2). Heating always involves weight loss or gain from volatilization of material (either natural or from thermal decomposition) and oxidation processes (oxygen absorption). However, considering biological variation in moisture content and for practicality, temperatures and conditions are selected to provide a compromise between volatilization degree and time of analysis (1).

Forced-draft or convection-type ovens may be used for drying (1). Ventilation from bottom to top is essential; top vents should remain open during analysis (12). Heat distribution must be uniform and temperature within ± 1.0°C or less. Uneven heat distribution can sometimes be overcome by altering heating element placement. Some laboratories utilize vacuum ovens (set ca 60°C) but excessive spattering can occur with some samples. This method does cause less heat damage to the sample however.

The most common error in this analysis arises from crust formation which traps moisture. Smaller samples (spread thinly) may be used to minimize the effect of crusting or the sample may be mixed with pre-dried sand or asbestos to provide a larger surface area. Sufficient numbers of samples should always be analyzed to compensate for anticipated variations.

a. Application

The following moisture determination by oven drying is applicable to all raw and cooked seafood products. If problems are encountered from fat spattering, excessive decomposition, or for samples of extremely low water content (oils), appropriate literature should be consulted (1, 9, 10).

b. Principle (10)

Representative portions of comminuted samples are dried to constant weight in an oven maintained at 103 ± 1°C.

c. Precautions

1. Samples must be protected from water loss before weighing. After comminution material for analysis should be stored in filled containers with close fitting lids. Freeze samples for long term storage and after defrost, mix thoroughly to re-distribute water which may have drained to the bottom.

2. If an oven is unavailable when samples are received or for excessive numbers of samples, material may be weighed into dishes and stored frozen until oven space becomes available. Prolonged drying time (over the weekend, etc.) may result in degradation and volatile loss.

3. Oven dried material readily absorbs moisture from the atmosphere; fish meal is particularly susceptible. Samples should be desiccated after removal from the oven and weighed immediately upon cooling.

4. Lipids absorb oxygen from the air and increase in weight on standing. High fat samples should be dried for the minimum time required.

5. Drying of high moisture or fat samples may be expedited by placing a disc of oven dried filter paper on the bottom of the drying dish before sample addition to spread and absorb the excess fluid and reduce spattering. Sometimes draping a pre-weighed filter paper disc over the sample will reduce loss by absorbing excess fluid and spattering fat.

6. Sample must be homogeneous and spread thinly (0.7 cm maximum) and evenly over the bottom of the drying dish.

7. If severe crusting is evident, drying time should be extended and in extreme cases the sample mixed with a small amount of pre-dried sand or asbestos fibres (caution-health hazard).

8. When dealing with atypical or new products, weight should be checked during the drying process to determine optimum drying time (to constant weight).

SAMPLE PREPARATION

Remove superfluous water (if not part of sample) by gently and briefly patting material with tissue or paper towel. Select representative specimens and comminute thoroughly. Homogeneity is absolutely necessary; if particles are present sample number should be increased. Comminuted samples which cannot be portioned immediately should be stored in filled closely covered containers. Freeze if storage of more than 24 hr is required, otherwise refrigeration is adequate. Before portioning, mix each sample thoroughly.

APPARATUS

1. **Drying dishes,** disposable aluminium moisture pans

2. **Drying oven** set at 103 ± 1°C, maintain good ventilation

3. **Balance** capable of weighing 0.001 g

PROCEDURE

1. Place pre-numbered (blunt pencil) empty moisture pans (three for each sample) in drying oven at 103°C for one hour. Cool in desiccator for 20 minutes.

2. Weigh each to nearest 0.001 g.

3. Mix comminuted sample well. Add 2-10 g to pan and spread evenly over bottom. Weigh pan and contents.

4. Dry in oven overnight, cool in desiccator, and weigh again. Samples may be checked for constancy of weight by returning to oven for 1 hour and reweighing.

CALCULATION

Moisture content of the samples, expressed as percent, is calculated as:

$$M = \frac{W_1 - W_2}{W_1 - W_0} \times 100$$

Where:

M = moisture content
W_1 = weight of dish and sample before drying
W_2 = weight of dish and sample after drying
W_0 = weight of dish (and filter paper, if any)

EXAMPLE

A sample of red hake flesh was dried overnight in a drying oven at 103°C. Weight of the drying dish and wet fish was 8.056 g, dried fish and dish was 2.446, and the dish itself was 1.141 g. By formula 1, the percentage moisture was:

2. MOISTURE

$$M = \frac{8.056 - 2.446}{8.056 - 1.141} \times 100$$

$$= \frac{5.610}{6.915} \times 100$$

$$= 81.1\%$$

REFERENCES

1. POMERANZ, Y. and C. E. Meloan, 1971. In Food Analysis: Theory and Practice AVI, Westport, Conn, pp 481-510.

2. MAKOWER, B., 1950. Determination of water in some dehydrated foods. In Advances in Chemistry Series 3. Am. Chem. Soc. Washington, D.C.

3. WARD, A. G., 1963. The nature of the forces between water and the macromolecular constituents of food. In Recent Advances in Food Science, vol. 3. J. M. Leitch, and D. N. Rhodes (Ed). Butterworths London.

4. LOVE, R. M., 1976. Processing cod: The influence of season and fishing ground. Torry Advisory Note No. 71, Torry Research Station, Aberdeen, Scotland.

5. SIMS, G. G. and C. E. Cosham, 1979. Collaborative study on a rapid method for the estimation of fat in herring - the official Norwegian method. Fisheries and Marine Service Technical Report No. 856.

6. DYER, W. J., D. F. Hiltz, E. R. Hayes, and V. G. Munro, 1977. Retail Frozen Fishery Products - Proximate and mineral composition of the edible portion. Can. Inst. Food Sci. Technol. J. 10:185.

7. WATT, B. K. and A. L. Merrill, 1963. Composition of foods. Agriculture handbook No. 8. U.S. Dept. of Agriculture.

8. MURRAY, J. and J. R. Burt. The composition of fish. Torry Advisory note No. 38, Aberdeen.

9. ANALYTICAL METHODS COMMITTEE, 1979. Recommended general methods for the examination of fish and fish products. Analyst 104:434.

10. "OFFICIAL AND TENTATIVE METHODS OF THE AMERICAN OIL CHEMISTS' SOCIETY", Vol. 1, Third Edition, AOCS, Champaign, IL, 1971.

11. KENT, M. 1982. A density independent microwave moisture meter for heterogeneous foodstuffs. J. Food Eng. 1:31.

12. WINDSOR, M. and S. Barlow. 1981. "Introduction to Fishery By-products". Fishing News Books Ltd., Farnham, England.

3. LIPID CONTENT (fat)

GENERAL DISCUSSION

The fat or lipid content of fish tissue, critical for determination of stability in frozen storage, adjustment of process requirements (canning) and nutritional labelling, varies tremendously with season and species. Lean fish (less than 1% fat) include cod, haddock, hake, pollock, and flatfish. Fatty fish include herring, mackerel, and salmon. Generally red flesh and the belly flap contain the highest proportion of fat. In salmon, fat content of belly flap vs. dorsal section can be 20-30% vs 5-10%, respectively.

The fat portion of muscle is comprised of a complex mixture of neutral lipids (triglycerides), polar lipids (phospholipids) and lesser components (sterols, sterol esters, free fatty acids, etc.). The proportion of each again is dependent on species and season. Therefore in order that fat extraction methods be applicable to all species, they must be versatile in their ability to extract each of these components. Traditional procedures involve drying and acid hydrolysis, exhaustive Soxhlet extraction with hexane or petroleum ether (1,2), or acid digestion and centrifugal separation and quantitation (3, 4). Since lipid recovered by these methods was not suitable for lipid composition studies (5), less harsh, more rapid processes involving acetone (6), chloroform/methanol (7,8) and 2:2:1.8 chloroform/methanol/water (5) extractions were developed and tested (9,10). One such procedure, the Bligh and Dyer method (5) which has been used extensively (sometimes with modifications (8)) was demonstrated in a collaborative study (11) to be precise, reproducible and accurate for quantitating fat content in processed and prepared foods for nutritional labelling, providing the chloroform, methanol and water were maintained in the reported proportions. This latter method will be described here.

a. Application

The Bligh and Dyer lipid extraction procedure (5) is applicable to the determination of total lipid in lean and fatty species of fish and fish products. Two methods are described: one for quantitative determination without total lipid recovery (method A) and a second (method B), for quantitative determination with complete recovery of lipid for subsequent analysis such as peroxide value, carbonyl value, fatty acid composition or free fatty acid determination. Method B yields a slightly lower fat content in some cases, 1 to 2% with fatty fish.

b. Principle:

Polar and non-polar lipids are extracted in a three step blending procedure in which chloroform, methanol, and water are maintained in the ratios of 1:2:0.8, 2:2:0.8, and 2:2:1.8. During the first two blending operations a single phase ternary solvent system is employed for complete extraction of tissue lipids. For further analysis, (method B) fat may be recovered completely by filtration through sodium sulfate (for total water removal) and freed of solvent by vacuum treatment.

c. Precautions:

1. For dry products adjust water content to conform to the required ratios during blending (see Method A, step 3).

2. For salted products (herring) use half the regular sample amount but do not decrease chloroform and methanol, i.e. in Method A, step 1 use 25 g sample with 100 ml methanol. Omit step 7 but add 75 ml (50 + 25) water to filtrate in step 9 after buchner funnel has been disconnected to achieve necessary chloroform, methanol, water ratio of 2:2:1.8.

3. With fatty species emulsions may form or difficulty may be encountered during filtration (method A, step 8). If this occurs and the condition cannot be rectified, repeat the extraction on fresh material modifying the procedure so that water addition (method A, step 7) occurs after filtration through the Buchner funnel (method A, step 9; method B, step 1)

4. Blending and filtration should be performed in a fume hood due to the health hazards posed by the solvents. Do not contact solvents with the skin.

SAMPLE PREPARATION:

Blend fresh or freshly thawed sample in a Cuisinart Food Processor with nitrogen flushing. Work quickly, do not expose sample to excess heat. If subsequent analyses (POV, COV, FFA) are not to be performed on the recovered lipid, nitrogen flushing is not necessary. Weigh 50 g portions of tissue into disposable plastic containers, flush with nitrogen and store on ice or preferably in freezer compartment of refrigerator until extracted (within 1 hour).

APPARATUS

1. **Waring blender** with 1 quart capacity jar, fitted with teflon gaskets between blade assembly and jar.

2. **Buchner funnel** ca 12 cm plate, with aspirator suction assembly, set in fume hood.

3. **Filter paper,** 11 cm, #4 Whatman or equivalent to fit Buchner funnel.

4. **Aluminum foil,** household quality.

5. **Pipetting bulb,** manual pipetting aid to fit 10 ml volumetric pipette.

6. **Drying oven** set at 103-105°C.

7. **Filter paper** (for method A): Whatman #1 and #4 or equivalent, 18.5 cm diameter.

8. **Rotary evaporator** (for method B), with nitrogen flush capability.

9. **Glassware:** (for method A) 500 ml graduated cylinders, 100 ml graduated cylinders, 10 ml volumetric pipette, and (additionally for method B) 500 ml separatory funnels, 500 ml round bottom flasks and glass filtering funnels.

REAGENTS:

1. **Chloroform,** technical grade for method A or ACS grade if continuing to method B.

2. **Methanol,** technical grade for method A or ACS grade if continuing to method B.

3. **Sodium sulfate (for method B only),** anhydrous granular ACS grade.

4. **Chloroform/methanol solution:** mix 100 ml chloroform with 100 ml methanol. Make fresh daily and store in a wash bottle.

PROCEDURE

Proceed with method A to step 9. Then continue for fat content only or apply method B if lipid is required for further analysis. For method B it may be convenient to increase sample size and solvent volumes.

METHOD A. Fat content only

1. In fume hood, add 50 g comminuted sample to blender jar with 100 ml methanol.

2. Add 50 ml chloroform to blender.

3. If extracting fish meal or other material of low water content (up to 20%), use 10 to 15 g sample and add 40 ml distilled water at this point; otherwise proceed to step 4. For salt fish see "Precautions" (note 2).

4. Form a cover for the blender jar of a double layer of aluminum foil. With foil extending to the outside of the jar, push center of foil down inside jar about 5 cm to form a baffle for the liquid and decrease spillover during blending.

5. Place regular cover on jar and blend for 2 minutes. Blend in fume hood.

6. Add 50 ml chloroform and blend an additional 30 seconds.

7. Add 50 ml distilled water and blend another 30 seconds.

8. Filter through Buchner funnel with aid of suction (water aspirator). Press cake with bottom of beaker to remove solvents. If filtration is difficult due to emulsion formation see "Precautions" section (note 3).

9. Rinse blender jar, funnel and press cake with 15 to 20 ml chloroform/methanol 1:1 mixture (from wash bottle).

10. Pour filtrate into 500 ml graduated cylinder. Rinse flask with 5-10 ml chloroform/methanol solution and add to cylinder.

11. After separation and clarification occurs (1 hr or overnight), record volume of lower chloroform layer.

12. With aspirator remove upper water/methanol layer completely. Some chloroform may be removed in the process.

13. Identify and preweigh to 0.001 g three aluminum weighing dishes for each chloroform extract (each cylinder).

14. In the fume hood and with the aid of a pipetting bulb, accurately pipet 10 ml chloroform solution into each of three preweighed aluminum weighing dish.

15. Allow chloroform to evaporate in fume hood until lipid residue remains (ca 2 h). Protect from dust and foreign materials.

16. Place drying dishes containing lipid residue in drying oven for 1 hour.

17. Remove dishes from oven, cool to room temperature in desiccator or on aluminum tray (not more than 15 minutes).

18. Carefully weigh aluminum dishes and collected lipid residue to the nearest 0.001 g.

METHOD B. Fat content with recovery for subsequent analysis:

1. Proceed as for method "A" to end of step #9, using ACS grade solvents and filtering through Buchner funnel.

2. Pour filtrate into 500 ml separatory funnel. Rinse flask with 5-10 ml chloroform/methanol solution.

3. Flush separatory funnel with nitrogen and stopper. Contents may be left overnight for complete separation or procedure may be continued once separation and clarification has occurred (at least 2 hours).

4. Fold #1 and #4 filter papers and place #4 inside #1. Pour sodium sulfate into filter paper cone until cone is three quarters filled. Place in glass funnel.

5. On retort stand assembly position separatory funnel so that stem extends approximately 1 cm into sodium sulfate.

6. Remove stopper from separatory funnel and allow lower chloroform layer to slowly pass through sodium sulfate into 500 ml round bottom flask (or 500 ml Erlenmeyer). A nitrogen tube may be introduced into collection flask to prevent oxygen pickup by solution. Stop flow of separatory funnel when chloroform layer has been exhausted.

7. Rinse sodium sulfate and exposed filter paper liberally (15-20 ml) with chloroform (ACS grade) from wash bottle to ensure complete recovery of lipid.

8. Transfer solution into preweighed round bottom flask. Attach to rotary evaporator connected to water aspirator and remove chloroform. Evaporator water bath temperature should not exceed 40°C. When complete, break vacuum (with nitrogen), reflush with nitrogen and protect from oxygen with preweighed ground glass stopper.

9. Correction for trace solvent: for further analysis trace solvent may be removed completely (method "b" below) or if the solvent will not interfere, the actual amount present may be estimated (method "a" below), a correction factor applied, and the lipid used accordingly.

   a. **Oven method:** If a sufficient quantity of lipid is available remove with the aid of a pasteur pipette two 0.5 g aliquots of crude lipid to pre-weighed disposable aluminum weighing dishes. Place dishes in a drying oven at 103-105°C for 1 hour, cool and weigh to nearest 0.001 g.

   b. **High vacuum method:** After rotary evaporation connect flask to high vacuum pump (with dry ice/acetone trap) for removal of trace amounts of solvent. For absolute accuracy step 9a above, (oven removal of trace solvents) may be applied to a small portion of lipid after disconnecting from vacuum pump.

CALCULATION: Percent lipid in sample

1. From method A, fat content (F) may be calculated as:

$$F = \frac{(W_2 - W_0) \times V_1 \times 100}{V_2 \times W_3} \qquad (1)$$

2. a. From method B, step 9a (oven removal of trace solvent) correction factor (f) and fat content (F) are:

$$f = \frac{(W_2 - W_0)}{(W_1 - W_0)} \qquad (2)$$

and

$$F = \frac{(W_5 - W_4) \times f \times 100}{W_3} \qquad (3)$$

Therefore weight of pure lipid in any aliquot removed is:

$$W_7 = W_6 \times f \qquad (4)$$

b. From method B, step 9b (high vacuum trace solvent removal) fat content (F) is:

$$F = \frac{(W_5 - W_4) \times 100}{W_3} \qquad (5)$$

Symbol Key: Efficiency of trace solvent removal can be checked by method B, step 9a and correction factor calculated by formula 2.

f = correction factor

F = percent (g/100 g) fat or lipid in tissue

V_1 = total volume (ml) of chloroform layer in graduated cylinder

V_2 = volume (ml) of chloroform aliquot removed to aluminum dish

W_0 = weight (g) of empty aluminum dish

W_1 = weight (g) of aluminum dish with crude lipid (added in step 9a of method B)

W_2 = weight (g) of aluminum dish with dried lipid residue

W_3 = weight (g) of tissue sample blended

W_4 = weight (g) of empty round bottom flask and stopper

W_5 = weight (g) of round bottom flask, stopper and lipid after high vacuum treatment

W_6 = weight (g) of lipid (with trace solvent) removed for subsequent analysis or testing

W_7 = true weight of lipid present in removed sample, corrected for trace solvent

EXAMPLE

A. A sample of salt herring received for analysis was comminuted and a 25.2 g (W_3) portion extracted with 100 ml methanol and 100 ml chloroform according to Method A but incorporating precaution 2. Seventy-five ml of water were added to the filtrate and the mixture transferred to the graduated cylinder. After 2 hrs. the volume of the lower chloroform phase was recorded as 108 ml (V_1), the top layer withdrawn and three 10 ml (V_2) aliquots removed to aluminum dishes weighing 0.983, 0.995 and 0.986 g (W_0). After drying respective weights of the dishes with residue were 1.238, 1.250, and 1.241 g (W_2). Using the first dish as an example (W_0 = 0.983, W_2 = 1.238), fat content of the herring (F) from formula 1 was:

$$F = \frac{(1.238 - 0.983) \times 108 \times 100}{10 \times 25.2}$$

$$= 10.9\%$$

B. A sample of mackerel fillets were comminuted and a 50 g portion (W_3) blended with 100 ml methanol, 100 ml chloroform, and 50 ml distilled water according to method A. After filtration (step 9) the filtrate was poured into a separatory funnel for overnight separation according to method B. The lower layer was then filtered through sodium sulfate and the chloroform removed from the extract by rotary evaporation. Weight of the empty round bottom flask and stopper was 163.49 g (W_4). After evaporation, flask, stopper and contents weighed 168.17 g (W_5). To determine trace solvent level a sample of lipid was withdrawn into an aluminum drying dish for oven drying (Method B, step 9a). Dish with and without lipid sample weighed 1.490 g (W_1) and 0.983 g (W_0), respectively. After drying dish with residue weighed 1.449 g (W_2). Correction factor from formula 2 is:

$$f = \frac{(1.449 - 0.983)}{(1.490 - 0.983)}$$

$$= 0.919$$

i.e. The lipid is 91.9% pure or contains 8.1 % solvent

Actual fat content of the mackerel from formula 3, therefore, is:

$$F = \frac{(168.17 - 163.49) \times 0.919 \times 100}{50}$$

$$= 8.60\%$$

In a subsequent analysis 2.5 g (W_6) of the lipid was withdrawn. Actual weight of lipid in the 2.5 g aliquot calculated from formula 4 is:

$$W_7 = 2.5 \times 0.919$$

$$= 2.30 \text{ g}$$

REFERENCES

1. ANALYTICAL METHODS COMMITTEE. 1979. Recommended general methods for the examination of fish and fish products. Analyst 104:434.

2. Official Methods of Analysis, 10th ed., Association of Official Agricultural Chemists, Washington, D.C., 1965. Sec. 22.037.

3. Official Methods of Analysis, 13th Ed. 1980. Association of Official Agricultural Chemists, Washington, D.C. Sec. 18.045.

4. SIMS, G.G., C.E. Cosham, and J.S. Kay. 1979. Determination of fat in herring using a modification of the AOAC Babcock method. Fisheries and Oceans Canada. New Series Circular No. 72.

5. BLIGH, E.G. and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37:911.

6. DAMBERGS, N. 1956. Acetone-water mixtures for the extraction and rapid estimation of fats of biological materials, particularly fish products. J. Fish. Res. Bd. Canada 13:791.

7. AMBROSE, M.E., B.J. Roche, and G.M. Knuble, Jr. 1969. Semimicro method for determining total lipids in fish meal. JAOAC 52:688.

8. HANSON, S.W.F. and J. Olley. 1963. Application of the Bligh and Dyer method of lipid extraction to tissue homogenates. Biochem. J. 83:101p

9. HUBBARD, W.D., A.J. Sheppard, D.R. Newkirk, A.R. Prosser and T. Osgood. 1977. Comparison of various methods for the extraction of total lipids, fatty acids, cholesterol, and other sterols from food products. J. Amer. Oil Chemists' Soc. 54:81.

10. MARINETTI, G.V. 1962. Chromatographic separation, identification and analysis of phosphatides. J. Lipid Res. 3:1.

11. DAUGHERTY, C.E. and H.G. Lento. 1983. Chloroform-methanol extraction method for determination of fat in foods: Collaborative study. J. Assoc. Off. Anal. Chem. 66:927.

4. PROTEIN - MICRO-KJELDAHL ANALYSIS

GENERAL DISCUSSION

The protein of living fish muscle is responsible for physical structure and activity; after death it becomes the component important to man for its nutritional and functional characteristics. Protein levels, averaging 15 to 20% in lean fish, vary both among and within species (1, 2).

Protein molecules, whether sarcoplasmic, structural, or connective are composed of arrays of amino acids each containing an amino ($-NH_2$) and carboxylic acid (-COOH) group. While there are only about twenty individual amino acids, the possibilities for their arrangement is almost limitless. This accounts for the broad spectrum of proteins which exist in nature (3).

Since fish protein is on the average 16% nitrogen, a total nitrogen analysis is a common approach to protein measurement. Other methods utilizing turbidity, physical complexing or analysis of certain groups on particular amino acids may suffer from variations when applied to various fish species.

Since the development of the organic nitrogen analysis in 1883 by the Danish scientist Kjeldahl, the method has undergone many changes (4, 5, 6, 7). In principle the sample is completely oxidized in strong acid until the protein nitrogen is reduced and transformed into ammonium sulfate. Ammonia is then liberated from the sulfate by addition of sodium hydroxide and distilled into boric or a standard acid where it may be titrated or quantified in some other manner.

The limiting or time consuming step in the analysis is the sulfuric acid digestion. In 1885, Wilforth found that digestion could be accelerated with certain catalysts and in 1889, Gunning suggested addition of potassium sulfate for boiling point elevation to achieve the same effect. The test is therefore sometimes called the Kjeldahl-Wilforth-Gunning method (4). Mercury is a superior catalyst for the digestion even though its inclusion requires the addition of sodium thiosulfate to decompose the mercury-ammonia complex formed (4). The strong oxidizer hydrogen proxide has been found to further speed digestion (8).

With all the developments and improvements to the Kjeldahl procedure the most common fault in carrying out the analysis continues to be failure to achieve complete digestion (9). Certain amino acids, tryptophan and histidine in particular, require extra long or harsh digestion conditions (4).

It must be noted that since the Kjeldahl analysis determines total nitrogen, commodities with excessively high levels of other compounds containing nitrogen will appear to contain erroneous high amounts of protein. Sources of such "non-protein" nitrogen can include TMAO, urea, taurine, peptides, amino acids, nucleotides, and related purine-based compounds (4, 10).

a. Application

The micro-Kjeldahl organic nitrogen determination for total protein (8) is applicable to all fishery products. Before the analysis is attempted, an estimate of protein must be made to determine appropriate sample size. Additionally since all organic nitrogen is analyzed as "protein", a non-protein nitrogen analysis (lab method #5) should be performed on the sample. A direct subtraction of protein and non-protein nitrogen is not always valid however since some protein components or "broken-down proteins", ie peptides and amino acids, are also included in the non-protein nitrogen analysis. The exact amount or subtraction error depends on the sample and is a function of biochemical make-up as well as enzymatic degradation occuring post-mortem.

b. Principle

Organic nitrogen present in the sample (protein and non-protein) is reduced and transformed to ammonium sulfate in the presence of sulfuric acid, catalysts and hydrogen peroxide; organic carbon and hydrogen are oxidized and the boiling solution is rendered

colorless. After a brief cooling period the digest is quantitatively transferred to a steam distillation apparatus and strong base is added to liberate the ammonia. Application of steam results in complete distillation of the ammonia into boric acid. The ammonia is in turn titrated from the boric acid with a strong acid (at low concentration) and the nitrogen quantified. Since protein is assumed to be 16% nitrogen, the factor 6.25 (ie. 100/16) is used to convert total nitrogen to total protein.

c. Precautions

1. Extreme caution must be exercised throughout the analysis with caustic chemicals, namely: concentrated sulfuric acid, hydrogen peroxide and sodium hydroxide. Severe burns may result.

2. Digestion flasks, the distillation apparatus and the water used for steam generation must be free from nitrogen; clean flasks with chromic acid. At least two blanks should be performed on the still before analysis begins. Only distilled water may be used for steam generation (be wary of commercial in-house steamlines).

3. Addition of hydrogen peroxide must occur dropwise and with caution. The mouth of the digestion flask should be directed into the fume hood for this addition step since the reaction of H_2O_2 is violent.

4. Digestion should be continued for 15 to 20 minutes after clarification to ensure complete protein degradation.

5. The digest must be cool before the water addition step and transfer to the distillation apparatus. Premature action will result in crystallization of catalysts in stopcock A (see Figure 4.1). This transfer must be quantitative.

6. The analysis is sensitive to all nitrogen containing compounds in the sample and in the atmosphere. Digestion flasks should not be exposed to areas where ammonia or other amines are being actively used.

7. For samples of extreme non-homogeneity it may be necessary to use the macro-Kjeldahl procedure and apparatus which utilizes a larger sample size. Consult the literature (4).

8. The sum of protein, fat, moisture and ash should equal one hundred percent. If this value is very much exceeded it is advisable to perform a non-protein nitrogen analysis (lab method #5).

SAMPLE PREPARATION

The sample must be representative and rendered as homogeneous as possible. Therefore extra comminuting or grinding may be necessary. It is strongly recommended that all weighing be performed at one time to prevent changes in moisture in the comminuted sample. Samples may be weighed directly or in nitrogen free paper and transfered to clean micro-Kjeldahl flasks and the tops sealed with parafilm until analysis time. If all analyses cannot be carried out the same day, preweighed samples may be stored frozen in Kjeldahl flasks or alternately wrapped in nitrogen-free paper in small vials. Do not expose samples to free ammonia or amines present in the atmosphere.

APPARATUS

1. **Distillation apparatus,** see Figure 4.1

2. **Digestion flasks,** 100 ml Kjeldahl flasks

3. **Digestion rack,** gas or electric

4. **Nitrogen free paper** (or cigarette papers)

5. **Glassware:** 4 x 125 ml Erlenmeyer flasks, 10 ml burette, 50 ml burette

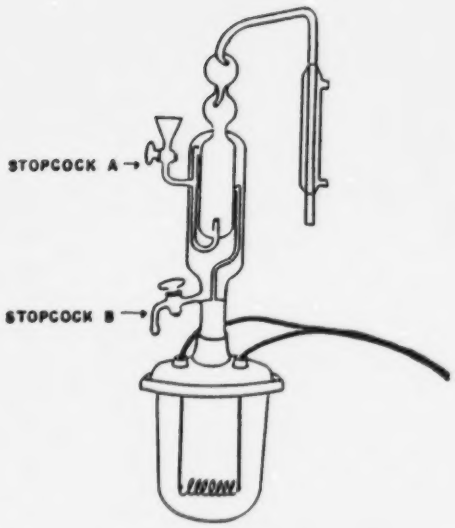


Figure 4.1 Micro-Kjeldahl distillation apparatus

REAGENTS

1. **Sulfuric acid** concentrated, N-free

2. **Catalyst:** Mix potassium sulfate (N-free) and mercuric oxide (N-free) in a ratio of 190:4 (w/w). For example, mix 190g K_2SO_4 with 4 g H_gO.

3. **Sodium hydroxide/sodium thiosulfate solution:** Dissolve 60 g NaOH and 5 g $Na_2S_2O_3$ $5H_2O$ in distilled water and dilute to 100 ml.

4. **Boric acid** saturated solution.

5. **Indicator solution** Use one of the following (a is preferred):

   a. MCB N-point indicator (BDH Chemicals Ltd.)

   b. Methyl red/methylene blue indicator: Mix 2 parts (100 ml) 0.2% methyl red solution (0.2 g methyl red dissolved in 100 ml ethanol) with 1 part (50 ml) 0.2% methylene blue solution (0.1 g methylene blue dissolved in 50 ml ethanol).

   c. Methyl red/bromocresol green indicator: Mix 1 part (50 ml) 0.2% methyl red solution (0.1 methyl red dissolved in 50 ml ethanol) with 5 parts (250 ml) 0.2% bromocresol green solution (0.5 g bromocresol green dissolved in 250 ml ethanol).

6. **Hydrochloric acid (0.02 N):** In a 1000 ml volumetric flask, dissolve 1.7 ml concentrated hydrochloric acid in approximately 700 ml of distilled water and bring up to volume. Standardize following section B of the procedure.

7. **Sodium hydroxide (0.05N):** In a 1000 ml volumetric flask, dissolve 2.0 g NaOH in approximately 700 ml distilled water and bring up to volume. Standardize following sectionn A of the procedure.

8. **Hydrogen peroxide,** 30%

9. **Phenolphthalein indicator** : Dissolve 1 g phenolphthalein in 100 ml ethanol (95%).

10. **Potassium acid phthalate,** ACS analytical grade, 5 g dried overnight at 103°C.

PROCEDURE

A. Standardization of 0.05N NaOH

1. Into each of three clean 250 ml Erlenmeyer flasks, weigh 0.30 g of dried potassium acid phthalate. Add 50 ml of distilled water (to dissolve) and 3 drops phenolphthalein indicator to each flask.

2. Fill a 50 ml buret with sodium hydroxide solution (ca 0.05N).

3. Titrate each potassium acid phthalate sample to the endpoint of the phenolphthalein indicator (first perceptible but permanent pink color) with 0.05N NaOH.

4. Record the position of the meniscus on the buret and the volume of NaOH used.

5. Calculate NaOH normality as:

$$N_1 = \frac{W_1 \times 1000}{204.22 \times V_1} \qquad (1)$$

where: N_1 = normality NaOH
V_1 = volume (ml) NaOH used for titration
W_1 = weight (g) potassium acid phthalate in flask

6. Repeat for each of the remaining phthalate samples and calculate average normality of the NaOH.

B. **Standardization of 0.02N HCl**

1. Pipette 25 ml aliquots of prepared HCl solution into each of 3 clean 125 ml Erlenmeyer flasks and 3 drops of phenolphthalein indicator.

2. Titrate each to the phenolphthalein endpoint (first perceptible but permanent (30 sec) pink color) with standardized 0.05 N NaOH (50 ml buret).

3. Record the volume of NaOH used for the titration.
Repeat for each of the three samples. Calculate the average volume of NaOH used; results should be within 0.03 ml. Calculate HCl normality as:

$$N_2 = \frac{N_1 \times V_2}{V_3} \qquad (2)$$

where: N_1 = normality NaOH
N_2 = normality HCl
V_2 = average volume (ml) NaOH used for titrations
V_3 = volume (ml) HCl added to each Erlenmeyer

C. **Digestion of sample**

1. Into a clean 100 ml micro-Kjeldahl digestion flask, weigh 2.3 g catalyst and 0.10 to 0.30 g sample wrapped in nitrogen free paper (to prevent spattering or loss of sample on neck of flask). Verify nitrogen content of paper.

2. Add 2.3 ml concentrated H_2SO_4 and swirl.

3. Heat flasks vigorously on digestion rack, until foam threatens to rise in the neck of the flask (15-30 sec).

4. Remove from heat and **carefully** add ca 1 ml hydrogen peroxide (slowly) down the side of the flask. **Caution** : Point the flask away when adding the H_2O_2, as sputtering and foaming may result.

5. Mix well by swirling and replace on heater. If solution doesn't clear, cool slightly and repeat addition of H_2O_2.

6. Heat mixture for an additional 15-20 minutes after solution has cleared to ensure complete digestion of proteinaceous material.

D. **Flushing of distillation apparatus**

1. Close stopcocks (or clamps) A and B on distillation apparatus

2. Place an Erlenmeyer flask with 50 ml of distilled water below condenser tube outlet; tube should not be in the water.

3. With steam generator resevoir about half filled with distilled water, turn on heat to initiate steam production.

4. When steam condensate begins to drip from condenser tube, turn off heat and immediately raise Erlenmeyer flask so that condenser stem is well below level of water.

5. As the apparatus cools, water from the flask will be drawn up the condenser tube into the main body of the distillation apparatus and then into the waste collection apparatus.

6. Open stopcock A and then stopcock B to drain waste collector.

7. Repeat entire procedure twice prior to distillation of samples.

E. **Distillation of sample**

1. Cool digested sample. Solids may form in the bottom of flask.

2. Add minimum quantity of distilled water (1-2 ml), swirl to dissolve solids, and transfer quantitatively to distillation apparatus through funnel above stopcock A (Figure 4.1).

3. Rinse flask 5 or 6 times with 1-2 ml portions of distilled water adding rinse to still.

4. Quickly add 10 ml $NaOH/Na_2S_2O_3$ solution to distillation apparatus. Immediately close stopcock (or clamp) A below funnel to prevent any escape of ammonia.

5. Begin steam generation. Collect distillate in a 125 ml Erlenmeyer flask containing 5 ml saturated boric acid and 4-6 drops of N-point indicator. Insure that tip of condenser is always below level of solution in Erlenmeyer.

6. Collect 15-20 ml of distillate.

7. Dilute to ca 50 ml with distilled water.

8. Immediately titrate boric acid with distillate to a gray endpoint (or the first appearance of violet) with 0.02 N HCl using an accurate 10 ml burette.

9. Titrate a blank which has been treated similarly to the sample, i.e. digested and distilled.

CALCULATION

Protein content, assuming a conversion factor of 6.25 from percent nitrogen from formula 3 is:

$$P = \frac{(V_4 - V_5) \times N_2 \times 14.007 \times 100 \times 6.25}{W_2 \times 1000} \quad (3)$$

where P =protein content as percent by weight
V_4=volume (ml) HCl to titrate the sample
V_5=volume (ml) HCl to titrate the blank
N_2=normality of HCl
W_2=weight (g) of sample

EXAMPLE

A 0.102 g (W_2) sample of cod flesh was analyzed for protein content. For titration, 9.73 ml (V_4) of 0.0215 N (N_2) HCl were used. The blank required 0.02 ml (V_5) HCl. The protein content (P), expressed as percent, may be calculated from formula 3 as:

$$P = \frac{(9.73-0.02) \times 0.0215 \times 14.007 \times 6.25}{0.102 \times 1000}$$

$$= \frac{9.71 \times 1.8}{.102 \times 1000} \times 100$$

= 17.1% protein

REFERENCES

1. SPINELLI, J., J.A. Dasson. 1982. Fish Proteins: Their modification and potential uses in the food industry. In Chemistry and Biochemistry of Marine Food Products (R.E. Martin, G.J. Flick, C.E. Hebard, and D.R. Ward, eds.) AVI Pub. Conn. pg 13-24.

2. DYER, W.J., D.I. Fraser, D.G. Ellis and W.A. MacCallum. 1957. Influence of intermittent short storage periods at 15°F, as encountered during refrigerator car transportation, on the quality of frozen cod stored at 0°F. J. Fish. Res. Bd. Canada 14:627.

3. MAHLER, H.R. and E.H. Cordes, 1966. Proteins: Classification, properties, purification. In Biological Chemistry, Harper and Row, New York.

4. POMERANZ, Y. and C.E. Meloan. 1971. In Food Analysis: Theory and Practice, pg 632.

5. BRADSTREET, R.B. 1940. A review of the Kjeldahl determination of organic nitrogen. Chem. Rev. 27:331.

6. BRADSTREET, R.B. 1965. The Kjeldahl Method for Organic Nitrogen. Academic Press, New York.

7. NEILL, C.D. 1962. The Kjeldahl protein test. Cereal Sci. Today 7:6-8,10,12.

8. CONCON, J.M., and Soltess, D. 1973. Rapid micro Kjeldahl digestion of cereal grains and other biological materials. Analytical Biochemistry 53:35.

9. HANSEN, H.E. 1981. Some notes on the ordinary Kjeldahl determination of protein in fish meal. In "Introduction to Fishery Products" (ed. M. Windsor and S. Barlow), Fishing News Books Ltd., Surrey, England. pp 142-147.

10. KONOSU, S., K. Watanabe and T. Shimizu. 1974. Distribution of nitrogenous constitutents in the muscle extracts of eight species of fish. Bull. Jap. Soc. Sci. Fish. 40:909.

5. NON-PROTEIN NITROGEN

GENERAL DISCUSSION

Total organic nitrogen is most commonly used to quantitate protein (1). However the presence of other nitrogenous compounds may sometimes indicate erroneously high protein; trimethylamine oxide, trimethylamine, dimethylamine, urea, taurine, peptides, amino acids, nucleotides, and related purine-based compounds may be included in the Kjeldahl nitrogen analysis (2, 3). While these compounds have often been related to the flavour of fish (4) they are not protein. Non-protein nitrogen (NPN) by definition arises from components that are not precipitable by 5% trichloroacetic acid and comprise between 0.5 and 1% of total weight of muscle (3, 5).

Levels of 5 to 10% TCA appear to have been used in the literature for extraction of non-protein nitrogen compounds (4); a strong protein denaturant is necessary. Analysis of nitrogen in the TCA extract is by micro-Kjeldahl analysis similar to laboratory method 4. In samples where low NPN is found, unruly volumes (up to 15 ml) of extract must often be used for the digestion.

a. Application

The non-protein nitrogen (NPN) analysis is applicable to all fishery products. Since it is difficult to estimate NPN before the analysis, trials may be necessary to establish the necessary "range" of sample size.

b. Principle

Protein is precipitated by trichloroacetic acid and the soluble nitrogenous compounds in the filtrate analyzed by micro-Kjeldahl analysis (see laboratory method 4, protein).

c. Precautions

1. Extreme caution must be exercised through the analysis with caustic chemicals; namely: concentrated sulfuric acid, hydrogen peroxide and sodium hydroxide. Severe burns may result.

2. Digestion flasks, the distillation apparatus and water used for steam generation must be free from nitrogen compounds; only distilled water may be used for steam generation. Clean flasks with chromic acid. A minimum of two blanks should be performed on the still before analysis begins.

3. Addition of hydrogen peroxide must occur dropwise and with caution. The mouth of the digestion flask should be directed into the fume hood for this addition step since the reaction of H_2O_2 is violent.

4. Digestion will be prolonged if a large volume of sample is required. Volume must be gradually reduced by boiling (with acid) on medium to low heat setting to ca 10 ml before peroxide and before peroxide addition and before digestion can be considered complete. Excessive heating will result in bumping of the sample.

5. The digest should be cool before addition of water. Premature transfer will result in crystallization of catalysts in stopcock A (see Figure 4.1). This transfer must be quantitative.

6. This analysis is sensitive to all nitrogen containing compounds in the sample and in the atmosphere. Digestion flasks should not be exposed to areas where ammonia or other amines are being actively used.

7. For powdered samples (fish meal) a larger volume of TCA may be necessary than the prescribed 1:2 extraction. The minimum quantity of TCA should be used to avoid dilution and thus the necessity for large digestion volumes.

8. Non-protein nitrogen should be subtracted from protein nitrogen only after careful consideration. For example, the NPN analysis measures free amino acid and peptide nitrogen which for nutritional purposes must still be included as "protein".

SAMPLE PREPARATION

The sample must be representative and well comminuted (food processor). Dry samples may require a larger amount of TCA than prescribed but the amount should be increased only to that absolutely necessary to avoid dilution as mentioned above.

APPARATUS

1. **Blender or food processor**
2. **Low speed centrifuge optional**
3. **Glass wool**
4. **Distillation apparatus,** see figure 4.1 in laboratory method 4, protein.
5. **Digestion flask** 100 ml Kjeldahl
6. **Digestion rack** gas or electric
7. **Glassware:** 125 ml Erlenmeyer flasks, accurate 10 ml burette, 50 ml burette

REAGENTS

1. **Sulfuric acid** concentrated, N-free
2. **Catalyst:** Mix potassium sulfate (N-free) and mercuric oxide (N-free) in a ratio of 190:4 (w/w). For example, mix 190g K_2SO_4 with 4 g H_gO.
3. **Sodium hydroxide/sodium thiosulfate solution:** Dissolve 60 g NaOH and 5 g $Na_2S_2O_3$ $5H_2O$ in distilled water and dilute to 100 ml.
4. **Boric acid** saturated solution.
5. **Indicator solution** Use one of the following (a is preferred):
   a. MCB N-point indicator (BDH Chemicals Ltd.)
   b. Methyl red/methylene blue indicator: Mix 2 parts (100 ml) 0.2% methyl red solution (0.2 g methyl red dissolved in 100 ml ethanol) with 1 part (50 ml) 0.2% methylene blue solution (0.1 g methylene blue dissolved in 50 ml ethanol).
   c. Methyl red/bromocresol green indicator: Mix 1 part (50 ml) 0.2% methyl red solution (0.1 methyl red dissolved in 50 ml ethanol) with 5 parts (250 ml) 0.2% bromocresol green solution (0.5 g bromocresol green dissolved in 250 ml ethanol)
6. **Hydrochloric acid (0.02 N):** In a 1000 ml volumetric flask, dissolve 1.7 ml concentrated hydrochloric acid in approximately 700 ml of distilled water and bring up to volume. Standardize following section B of the procedure.
7. **Sodium hydroxide (0.05N):** In a 1000 ml volumetric flask, dissolve 2.0 g NaOH in approximately 700 ml distilled water and bring up to volume. Standardize following sectionn A of the procedure.
8. **Hydrogen peroxide,** 30%
9. **Phenolphthalein indicator** : Dissolve 1 g phenolphthalein in 100 ml ethanol (95%).
10. **Potassium acid phthalate,** ACS analytical grade, 5 g dried overnight at 103°C.
11. **Trichloroacetic acid** (10%): Dissolve 10.0 g TCA in 90.0 ml distilled water.

PROCEDURE

A. Standardization of 0.05N NaOH

1. Into each of three clean 250 ml Erlenmeyer flasks, weigh 0.30 g of dried potassium acid phthalate. Add 50 ml of distilled water (to dissolve) and 3 drops phenolphthalein indicator to each flask.

2. Fill a 50 ml buret with sodium hydroxide solution (ca 0.05N).

3. Titrate each potassium acid phthalate sample to the endpoint of the phenolphthalein indicator (first perceptible but permanent pink color) with 0.05N NaOH.

4. Record the position of the meniscus on the buret and the volume of NaOH used.

5. Calculate NaOH normality as:

$$N_1 = \frac{W_1 \times 1000}{204.22 \times V_1} \qquad (1)$$

where: N_1 = normality NaOH
V_1 = volume (ml) NaOH used for titration
W_1 = weight (g) potassium acid phthalate in flask

6. Repeat for each of the remaining phthalate samples and calculate average normality of the NaOH.

B. Standardization of 0.02N HCl

1. Pipette 25 ml aliquots of prepared HCl solution into each of 3 clean 125 ml Erlenmeyer flasks and 3 drops of phenolphthalein indicator.

2. Titrate each to the phenolphthalein endpoint (first perceptible but permanent (30 sec) pink color) with standardized 0.05 N NaOH (50 ml buret).

3. Record the volume of NaOH used for the titration.
Repeat for each of the three samples. Calculate the average volume of NaOH used; results should be within 0.03 ml.

Calculate HCl normality as:

$$N_2 = \frac{N_1 \times V2}{V_3} \qquad (2)$$

where: N_1 = normality NaOH
N_2 = normality HCl
V_2 = average volume (ml) NaOH used for titrations
V_3 = volume (ml) HCl added to each Erlenmeyer

C. TCA Extraction of Sample

1. Blend sample in blender or food processor to a fine paste.

2. Weigh 50 g samples and freeze until ready to extract.

3. Add 50 g samples and 100 ml 10% TCA (1:2 extraction) to a small blender jar and blend thoroughly.

4. Centrifuge at 4°C for 15 minutes at about 2000 x g (approximately 4000 rpm). An alternate purification procedure to centrifugation and decanting is filtration through Whatman #4 filter paper.

5. Decant supernatant fluid through glass wool.

6. Extracts may be frozen at -20°C for extended periods of time with little deterioration.

D. Digestion of TCA Extracts

1. Into a clean 100 ml micro-Kjeldahl digestion flask, place 2.3 g catalyst and 1 to 4 ml TCA extract (volume depends on nature of sample; for greater volumes see precaution #4).

2. Add 2.3 ml concentrated H_2SO_4 and swirl.

3. Rapidly heat flasks on digestion rack, until foam threatens to rise in the neck of the flask (15-30 sec). If sample volume is high extend heating time to facilitate evaporation of excess liquid.

4. Remove from heat and **carefully** add ca 1 ml hydrogen peroxide down the side of the flask. **Caution** : Point the flask away when adding the H_2O_2, as sputtering and foaming may result.

5. Mix well by swirling and replace on heater. If solution doesn't clear, cool slightly and repeat addition of H_2O_2.

6. Heat mixture for an additional 15-20 minutes after solution has cleared to ensure complete digestion.

E. Flushing of Distillation Apparatus

1. Close stopcocks (or clamps) A and B on distillation apparatus

2. Place an Erlenmeyer flask with 50 ml of distilled water below condenser tube outlet; tube should not be in the water.

3. With steam generator resevoir about half filled with distilled water, turn on heat to initiate steam production.

4. When steam condensate begins to drip from condenser tube, turn off heat and immediately raise Erlenmeyer flask so that condenser stem is well below level of water.

5. As the apparatus cools, water from the flask will be drawn up the condenser tube into the main body of the distillation apparatus and then into the waste collection apparatus.

6. Open stopcock A and then stopcock B to drain waste collector.

7. Repeat entire procedure twice prior to distillation of samples.

F. Distillation of Sample

1. Cool digested sample. Solids may form in the bottom of flask.

2. Add minimum quantity of distilled water (1-2 ml), swirl to dissolve solids, and transfer quantitatively to distillation apparatus through funnel above stopcock A (Figure 4.1).

3. Rinse flask 5 or 6 times with 1-2 ml portions of distilled water adding rinse to still.

4. Quickly add 10 ml $NaOH/Na_2S_2O_3$ solution to distillation apparatus. Immediately close stopcock (or clamp) A below funnel to prevent any escape of ammonia.

5. Begin steam generation. Collect distillate in a 125 ml Erlenmeyer flask containing 5 ml saturated boric acid and 4-6 drops of N-point indicator. Insure that tip of condenser is always below level of solution in Erlenmeyer.

6. Collect 15-20 ml of distillate.

7. Dilute to ca 50 ml with distilled water.

8. Immediately titrate boric acid with distillate to a gray endpoint (or the first appearance of violet) with 0.02 N HCl using an accurate 10 ml burette.

9. Titrate a blank which has been treated similarly to the sample, i.e. digested and distilled.

CALCULATION

Nonprotein nitrogen, expressed as percent nitrogen:

$$NPN = \frac{(V_4-V_5)xN_2x14.007x[V_6+(0.01xMxW_2)]}{V_7xW_2x10} \quad (3)$$

where:

M = moisture content of sample (percent)
N_2 = normality of HCl
NPN= non protein nitrogen as percent by weight
V_4 = volume (ml) HCl to titrate sample
V_5 = volume (ml) HCl to titrate blank
V_6 = volume (ml) TCA added for 1:2 extraction
V_7 = volume (ml) TCA extract added to digestion flask
W_2 = weight (g) of fish used in 1:2 extraction

EXAMPLE

Fifty grams (W_2) of cod was extracted with 100 ml (V_6) TCA. Exactly 1.1 ml (V_7) of extract was digested in the Kjeldahl apparatus. The distillate was titrated with

4.16 ml (V_4) of 0.0217 N (N_2) HCl; the blank required 0.01 ml (V_5). Assuming a moisture content of 80% (M), the percent nonprotein nitrogen (NPN) in the sample, expressed as percent nitrogen from formula 3 is:

$$NPN = \frac{(4.16-0.01)\times 0.0217\times 14.007\times[100+(0.01\times 80\times 50)]}{1.1\times 50\times 10}$$

$$= \frac{4.15\times 0.304\times 140}{550}$$

= 0.321% nonprotein nitrogen

REFERENCES

1. POMERANZ, Y. and C.E. Meloan. 1971. In Food Analysis: Theory and Practice, pg 632.

2. KONOSU, S., K. Watanabe and T. Shimizu. 1974. Distribution of nitrogenous constitutents in the muscle extracts of eight species of fish. Bull. Jap. Soc. Sci. Fish. 40:909.

3. SPINELLI, J., J.A. Dasson. 1982. Fish Proteins: Their modification and potential uses in the food industry. In Chemistry and Biochemistry of Marine Food Products (R.E. Martin, G.J. Flick, C.E. Hebard, and D.R. Ward, eds.) AVI Pub. Conn. pg 13-24.

4. JONES, N.R. 1963. Chemical changes in fish muscle during storage. Proc. Nutr. Soc. 22:172.

5. DYER, W.J., D.I. Fraser, D.G. Ellis and W.A. MacCallum. 1957. Influence of intermittent short storage periods at 15°F, as encountered during refrigerator car transportation, on the quality of frozen cod stored at 0°F. J. Fish. Res.Bd. Canada 14:627.

6. ASH CONTENT

GENERAL DISCUSSION

Ash is the inorganic residue remaining after incineration of biological material. Composition of the ash will depend on raw material (nature, degree of processing, contamination, inorganic additives) and method of ashing (1). The procedure is used to assess the total mineral content of a sample or as a first step for further analysis of specific elemental components. Two methods are commonly used: dry ashing in which a precharred sample is heated in the presence of air at temperatures ranging from 400 to 700°C (usually 550°C) and wet ashing, where samples are digested in the presence of strong oxidizing acids. Since certain elements, particularly trace elements, are volatilized in the dry ash process or are absorbed by the ashing vessels, wet ashing is necessary for certain analysis. However, for total mineral content of foods, dry ashing in porcelain crucibles is the most common procedure and completely adequate. The ash content of the edible portion of fresh fish ranges from 1 to 2% (1).

a. **Application**

The dry ash procedure is applicable to all fishery products and by-products of varying moisture and fat levels. Procedures for low (intermediate) and high moisture samples are presented.

b. **Principle**

Ash is the inorganic residue remaining after incineration at 550°C. Volatile components and all carbon are removed in the process and elements are reduced to their most stable form, usually oxides or sulfates.

c. **Precautions**

1. High moisture samples must be dried before ashing.

2. Caution must be exercised to avoid contamination of the ash with foreign material (from the inside of the furnace) or loss of the delicate ash particles.

3. Heat high lipid containing samples slowly to prevent spattering of the fat with loss of sample or cross-contamination.

4. Remove hot crucibles from oven with caution to avoid burns.

5. Place hot vessels on an asbestos mat and then into a desiccator with the lid slightly ajar to prevent lid bumping from air expansion.

6. Continue ashing until all carbonaceous material is removed, ie. ash is creamy white in color.

SAMPLE PREPARATION

Sample must be rendered homogeneous before analysis. If skin and bones are present and analysis is to include all of the material, then sample must be comminuted by passing three times through a hand grinder. If any liquid separates, mix throughly before weighing sample. For liquid samples stir vigorously before pouring or portioning. If particulate matter exists, material should be homogenized in blender or by Polytron (Brinkman Instruments, Rexdale, Ontario) before sampling.

APPARATUS

1. **Electric muffle furnace,** to be maintained at 550°C.

2. **Porcelain crucibles,** 30 ml, 43x37 mm (diameter x height).

3. **Glass rod,** with flattened end.

4. **Tongs,** long handled metal

PROCEDURE

METHOD A. Low and intermediate moisture samples (2)

1. Heat crucibles and covers in a muffle furnace at 550°C for a minimum of 1 hour, or overnight. Cool in a desiccator and weigh (with covers) to the nearest 0.0001 g.

2. Add 4 g samples into each crucible and weigh with cover to the nearest 0.0001 g.

Crucibles should be one half to three quarters full.

3. Carefully char material on an electric hot plate or with a Bunsen burner (low flame) taking care not to allow sample to burst into flame. If flaming occurs quickly cover crucible with cover. N.B. Pre-charring may be omitted if samples are placed in a cold muffle furnace and temperature is raised gradually.

4. Place covered crucibles with samples in a cold muffle furnace. Set furnace to 550°C and leave overnight.

5. With tongs carefully remove crucibles from furnace and allow to cool in desiccator. Do not close desiccator completely; allow heated air to escape.

6. If ash appears creamy white in color and free of black particles weigh the crucible with cover.

7. Carefully pulverize ash with a clean dry glass rod taking care not to lose any sample. If black particles are observed in crushed material rinse glass rod with 1-2 ml of distilled water and evaporate to dryness in a hot plate again taking care not to lose any ash. Return covered crucibles to furnace at 550°C until ash becomes white, probably overnight. Cool and re-check color.

8. Weigh covered crucible (and contents) to nearest 0.0001 g.

METHOD B. **High moisture samples** (3)

1. Heat crucibles and covers in a muffle furnace at 550°C for a minimum of 1 hour, or overnight. Cool in a desiccator and weigh (with covers) to nearest 0.0001 g.

2. Add 6 g sample into each crucible and weigh to nearest 0.0001 g. With covers off, place crucibles in drying oven set at 103°C overnight to remove moisture.

3. Proceed as in step 3 of preceding "low/ intermediate moisture" section and continue analysis.

CALCULATION

Ash content is:

$$A = \frac{(W_3 - W_1) \times 100}{W_2 - W_1} \qquad (1)$$

where A = percent ash

W_1 = weight of empty crucible with cover

W_2 = weight of crucible, cover and sample added

W_3 = weight of crucible, cover and ash

EXAMPLE

A sample of minced cod frames was analyzed for ash content. The crucible with cover weighed 29.8311 g (W_1); crucible, cover and sample were 35.6944 (W_2). After ashing, the crucible, cover and ash weighed 30.2425 g (W_3). Ash content (A) from formula (1) is:

$$A = \frac{(30.2425 - 29.8311)}{(35.6944 - 29.8311)} \times 100$$

$$= 7.02\%$$

REFERENCES

1. POMERANZ, Y. and C.E. Meloan. 1971. "Food Analysis, Theory and Practice." AVI Publishing Co., Wesport, Conn.

2. WINDSOR, M. and S. Barlow. 1981. "Introduction to Fishery By-products". Fishing News Books Ltd., Farnham, England, pp 150-151.

3. Official Methods of Analysis, 13th Ed. 1980. Association of Official Agricultural Chemists, Washington, D.C. Sec. 18.040.

7. SALT CONTENT

GENERAL DISCUSSION

Salt has been applied to the preservation of meat and fish for a considerable time in the history of mankind (1). Cod, herring and mackerel are still popular in a salted form and with the development of rapid salting processes (2) many of the unattractive species often discarded as by-catch are being investigated for their salting potential (3).

Salt content of salted cod fish can vary from 4 to 20% wet weight before drying (4). While there appears to be a definite relation between water loss and salt pickup (4) water/salt transfer is a function of brine strength (5, 6) and temperature (4, 7).During salting a swelling occurs in the muscle (6) and eventually the proteins are "coagulated" (3), "salted out" (6) or "denatured" (4).

The measurement of salt is a prerequisite for quality control in the production of salt fish products, especially for process modification. Salt content is also important for dietary reasons (8).

Procedures for salt determination have involved dichlorofluorescein (9); addition of silver nitrate to precepitate silver chloride with thiocyanate back titration of excess silver nitrate (10, 11, 12); potentiometric titration with silver nitrate (12); application of Quantab chloride titrator indicating strips (Ames Co., Division of Miles Laboratories Inc.) (12); and conductivity measurements. One of the most rapid and convenient procedures for salt measurement in fishery products is the conductivity method.

a. Application

The conductivity method for salt determination is applicable to all fishery products with salt content greater than approximately 0.5%.

b. Principle

The resistance offered by an aqueouos medium to the flow of electricity varies in a manner inversely proportional to the concentration of dissociating inorganic salts. This principle forms the basis of the conductivity procedure for measurement of salt content in fishery products.

The method involves blending a sample with water, measuring the electrical conductivity in milli-mho units of the solution by means of a conductivity meter and interpreting the results from standard curves.

c. Precautions

1. Temperature control is critical for measurement; all measured solutions must be at the same temperature as standards used for the preparation of the standard curve.

2. For samples of low salt content the proportion of water must be decreased. However, in the extreme, some error may be encountered from the presence of natural salts since the procedure is not specific for sodium but rather takes into account all inorganic ionizable salts. For low concentrations, the silver nitrate titration procedure (12) may be preferable.

SAMPLE PREPARATION

1. With a sharp knife cut sample into portions of approximately 1/2" x 1/2".

2. Comminute sample:

   a. For lean fish (salt cod), place several portions into a dry blender jar and blend for 10 second intervals until material is shredded.

   b. For fatty fish (herring, mackerel), comminute sample in a food processor until a homogeneous paste has been produced. If portions are very dry a blender may be used for comminution.

3. Pre-weigh portions of fish according to anticipated salt content, i.e. 10, 20, or 40 g portions for 18, 8 and 4% NaCL (wet weight) respectively.

4. Save some material for moisture determination.

APPARATUS

1. **Conductivity meter** equipped with conductivity cell. For example, Radiometer CDM2 meter with CDC-114 flow cell is appropriate (see Figure 7.1).

2. **Blender,** Waring or equivalent

3. **Water bath,** constant temperature, maintained at 20 or 25 ± 0.5°C.

4. **Glass wool** or filter paper, Whatman #4

5. **Glassware:** 100 ml measuring cylinders, funnels (glass or plastic), test tubes (18 x 150mm), 100 ml beaker, 100 ml volumetric flasks.

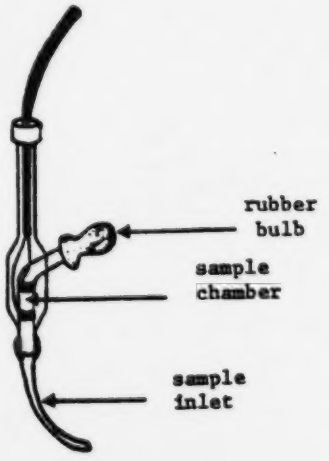


Figure 7.1 Conductivity cell

REAGENTS

1. **Sodium chloride:** Dry 30 g ACS grade NaCl at 110°C overnight in 100 ml beaker and store in desiccator.

2. **Sodium chloride standards:** To 100 ml volumetric flasks add 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6 and 1.8 g portions (accurately weighed) of NaCl with 80 ml distilled water. Swirl to dissolve and make up to volume. Standards represent 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6 and 1.8% w/v solutions.

PROCEDURE

A. **Calibration of meter**

1. Add standard solutions to clean test tubes and equilibrate in constant temperature bath - allow at least 15 minutes.

2. With attached bulb rinse cell with distilled water. Cell should be stored filled with distilled water or soaked for at least 30 minutes before use.

3. With function switch on "calibrate" and range at 5 milli-mho, set pointer to red calibration mark on meter.

4. Partially fill cell with standard solution of lowest salt concentration and dispel into waste container.

5. Fill and empty (back into test tube) cell several times with standard solution to ensure temperature equilibration of cell. Allow cell to rest in test tube (in bath).

6. Record reading from meter.

7. Proceed (without rinsing) to solution of next higher concentration, discarding first aliquot drawn. If meter goes off scale switch to 15 milli-mho range.

B. **Salt in Sample**

1. Determine moisture of samples as described in Laboratory Method 2.

2. In a Waring blender blend preweighed samples of fish with 200 ml distilled water for 1 to 2 minutes until homogeneity is achieved.

3. Filter a portion of the blend through glass wool or Whatman # 4 filter paper into a glass test tube to remove particles.

4. Place test tube in water bath at set temperature and allow at least 15 minutes for equilibration.

5. Rinse cell with distilled water at temperature of water bath; cell should always be stored partially filled with distilled water.

6. On conductivity meter set output to 5 milli-mho and function switch to "calibrate". With calibration knob adjust meter so that needle rests on red calibration mark. Set switch to "measure".

7. With rubber bulb attached to cell partially fill cell chamber with filtered sample solution to rinse. Dispel into waste beaker.

8. Refill chamber with new solution ensuring that coil in cell is immersed in solution. Leave cell in the test tube (in water bath)and fill and empty cell several times to ensure constancy of and homogeneity of solution. Record reading from meter. If reading is off scale, set meter to 15 milli-mho. Dispel solution.

9. Rinse cell chamber with distilled water and proceed to next solution.

10. If readings are too low or extremely high, sample weight should be adjusted and the analysis repeated.

CALCULATIONS:

A. Standard Curve

On graph paper plot meter reading vs g NaCl/100 ml as shown in Figure 7.2 as follows:

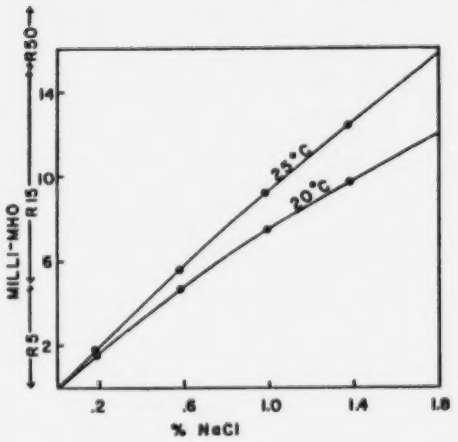


Figure 7.2 Typical conductivity curve of conductivity vs salt concentration

B. Salt Concentration in Samples

From the meter reading obtained for each solution determine from the calibration graph a final NaCl concentration.

Salt content in sample may be calculated as:

$$C = \frac{\frac{100R}{W}\left(V + \frac{M \times W}{100}\right)}{100}$$

$$= \frac{R}{W}\left(V + \frac{M \times W}{100}\right) \qquad (1)$$

Where:

C = concentration of NaCl in sample expressed as percent on a wet weight base
M = moisture as percent by weight
R = % NaCl reading determined from graph
V = volume (ml) distilled water added
W = weight (g) of sample used

EXAMPLE

Salt cod estimated at 10% salt content was blended and preweighed into 20 g (W) portions. Moisture content was found to be 72% (M). After blending with 200 ml (V) water and equilibration to 25°C, a value of 7.8 was obtained from the meter, which from the graph (Figure 7.2) is equivalent to 0.815 g NaCl/100 ml (R). Salt content from formula 1 is:

$$C = \frac{0.815}{20}\left(200 + \frac{72 \times 20}{100}\right)$$

$$= 8.74\% \text{ NaCl}$$

REFERENCES

1. DELVALLE, F.R., and J.T.R. Nickerson. 1967. Studies on salting and drying fish. I. Equilibrium Considerations in salting. J. Food Sci. 32:173.

2. WOJTOWICZ, M.B., M.G. Fierheller, R. Legendre, and L.W. Regier. 1977. A technique for salting lean minced fish. Fisheries and Marine Service Technical Report No. 731.

3. YOUNG, R.H., E. Coria, E. Cruz, and J. Baldry. 1979. Development and acceptability testing of a modified salt fish product prepared from shrimp by-catch. J. Fd. Technol. 14:509.

4. CREAN, P.B. 1961. The light pickle salting of cod. J. Fish Res. Bd. Canada 18:833.

5. FOUGERE, H. 1952. The water transfer in codfish muscle immersed in sodium chloride solutions. J. Fish. Res. Bd. Canada 9:388.

6. HAMM, R. 1960. Biochemistry of meat rehydration. Advances in Food Research 10:414.

7. WATERMAN, J.J. 1976. The production of dried fish. FAO Fisheries Technical Paper No. 160.

8. WEKELL, J.C., F.M. Teeny, E.J.J. Gouglitz, L. Hathorn and J. Spinelli. 1983. Implications of reduced sodium usage and problems in fish and shellfish. Food Tech. 37(9):51.

9. DYER, W.J. 1943. Ind. Eng. Chem. Analytical Ed. 15:439.

10. Analytical Methods Committee. 1979. Recommended general methods for the examination of fish and fish products. Analyst 104:434.

11. WINDSOR, M. and S. Barlow. 1981. "Introduction to Fishery By-products". Fishing News Books Ltd., Farnham, England.

12. "Official and Tentative Methods of the American Oil Chemists' Society", Vol. 1, Third Edition, AOCS, Champaign, IL, 1971.

8. BONE CONTENT

GENERAL DISCUSSION

In the production of minced or deboned fish, process parameters must be critically controlled to avoid inclusion of bone and bone fragments. Measurement of bone content is necessary for optimization of efficiency of the deboning process and as a quality control measure. Some equipment, particulary in the meat industry is capable of completely disintegrating bone; in ground meat therefore, total calcium often serves as a measure of bone content. Deboners used in the fish processing industry are less comminuting and pass bones of a size visible to the naked eye (1). Methods based on flesh dissolution, or disintegration and physical separation of bone fragments usually suffice for analysis of bone in fish. Physical blending with decantation (2, 3), 1 molar sodium hydroxide with chloroform separation (4), and papain (5), pepsin (6) and urea digestion with washing (7) have been used successfully for flesh disintegration and recovery of foreign particles (bones, parasites).

a. Application

The digestion method is applicable to all fish flesh where bone fragments are to be isolated. The method of Yamamoto and Wong (7) described here is relatively mild and will allow recovery of fine bones such as those found in herring. For fatty fish (mackerel, herring) some of the lipid must be removed before digestion.

b. Principle

Flesh after fat removal (if necessary) is allowed to digest overnight (with gentle stirring) in urea/NaOH with tap water, facilitating collection of bones and fragments by filtration.

c. Precautions

Caution must be exercised in the flushing of dissolved material to ensure no loss of fine bone fragments.

SAMPLE PREPARATION

Most samples requiring bone content measurement will already be in minced form. If whole fillets are to be analyzed, they may be comminuted with a Cuisinant food processor fitted with a dough blade or alternately reduced with any non-grinding apparatus. Freeze material for long term storage; refrigerated temperatures may be used for the short term.

APPARATUS

a. **Magnetic stirrer:** capable of stirring a volume of 2000 ml.

b. **Filter paper:** # 4 Whatman or equivalent

c. **Glassware:** 1000 ml Erlenmeyer, 2000 ml Erlenmeyer flasks (graduated if possible), large glass funnels or a Buchner funnel assembly, glass stirring rod.

REAGENTS

a. **Urea, 3M in 0.02 M NaOH.** To approx. 1800 ml tap water in a 2000 ml graduated Erlenmeyer add 1.80 g urea, technical grade and 1.6 g NaOH, technical grade. Stir to dissolve and bring to the 2000 ml mark with water.

b. **Chloroform,** technical grade

c. **Methanol,** technical grade

PROCEDURE

A. **Fat fish - preliminary lipid removal**

1. To 100 g minced flesh in a 1000 ml Erlenmeyer add 200 ml methanol and 100 ml chloroform. Disperse flesh with a glass stirring rod.

2. Add 100 ml chloroform and continue stirring.

3. Decant solvents taking care not to lose bone fragments.

4. Add 100 ml methanol, swirl, and decant solvent.

5. Transfer flesh and all bone fragments to a 2000 ml Erlenmeyer flask. Proceed as for lean fish.

B. Lean fish

1. Add 100 g minced flesh to 2000 ml urea/NaOH solution. Adjust stirrer for gentle stirring.

2. With glass stirring rod break up large pieces to aid digestion.

3. Stir overnight at a rate to prevent settling of material.

4. Attach a small hose to a cold water tap and allow cold water to flow into the Erlenmeyer at a rate which allows dissolved material to be flushed from the flask yet retaining bones. Continue flushing until solution is clear and free of dissolved flesh.

5. Dry a disc of filter paper, Whatman # 4 or equivalent in a 103°C oven for 1 hour. Cool and weigh filter paper.

6. Decant as much water as possible from Erlenmeyer without losing bones and filter remainder, either by gravity or on a Buchner funnel with vacuum.

7. Dry filter paper at 105°C oven overnight, cool, and weigh paper and bones.

8. Bones may be sized after drying by passing through various sized screens.

CALCULATION

Percent bone by weight may be calculated as:

$$B = \frac{W_2 - W_1}{W_3} \times 100 \qquad (1)$$

where,

B = percent bone by weight
W_1 = weight (g) of filter paper
W_2 = weight (g) of filter paper with bones
W_3 = weight (g) of fish sample dissolved

EXAMPLE

A 150 g (W_3) sample of minced herring offal was withdrawn for bone content analysis. The dried filter paper disc weighed 1.642 g (W_1); after collection of bones and redrying weight was 4.827 g (W_2). Bone content (B) of the herring offal from formula (1) is:

$$B = \frac{(4.827 - 1.642)}{150} \times 100$$

$$= 2.12\%$$

REFERENCES

1. ANONYMOUS. 1980. General discussion. Third National Technical Seminar on Mechanical Recovery and Utilization of Fish. Raleigh, North Carolina, December 1-3, 1980.

2. PATASHNIK, M., G. Kudo and D. Miyauchi. 1974. Bone particle content of some minced fish muscle products. J. Food Sci. 39:588.

3. PATASHNIK, M., D. Miyauchi, and G. Kudo. 1974. Controlling bone particle content in minced fish muscle. Marine Fisheries Review 36:37.

4. DINGLE, J.R., W.D. Aubut, D.W. Lemon, and W. Robson. 1974. The measurement of the bone content in minced fish flesh. Fisheries and Marine Service, Canada, New Series Circular No. 44.

5. HILL, R.M. and B.D. Hites. 1968. Meat and meat products. Determination of small bone particles in meat. J. Am. Oil Chemists' Soc. 51:1175.

6. WANG, J.C.C., and S. Varga. 1977. Mechanical removal and determination of cod worm (Phocanema decipiens) from fish flesh. Department of Fisheries and the Environment, Canada. New Series Circular No. 65.

7. YAMAMOTO, M. and J. Wong. 1974. Simple chemical method for isolating bone fragments in minced fish flesh. J. Food Sci. 39:1259.

9. TRIMETHYLAMINE (TMA)

GENERAL DISCUSSION

Most marine species contain trimethylamine oxide (TMAO) for osmoregulation (1). The elasmobranch (sharks) and gadoid (cod, pollock, hake) families have particularly high levels of TMAO; actual concentrations vary with species, stock, area, and time of year (2, 3, 4).

During ambient chilled or iced storage of marine fish, TMAO is reduced to the odoriforous compound trimethylamine (TMA) by facultative bacteria, likely Pseudomonas (or Alteromonas) putrefaciens (2, 5, 3). Although several species of bacteria are capable of this reduction, 80% of all TMA producers are pseudomonads; TMA levels often bear a linear relationship to their numbers (5). An excellent review of TMAO, TMA and DMA occurrence and significance has been presented by Hebard et al. (3).

TMA has often been used as a measure of bacterial spoilage in fish and several attempts have been made to correlate its level with organoleptic quality (6, 7, 8, 9, 10). It has been reported however, that with different initial levels of TMAO, TMA accumulates at different rates for different species (11, 12, 13, 14). For example, TMA levels were higher in cod than in haddock when fish were of comparable organoleptic quality (11, 12). Seasonal fluctuations were also shown to have an effect on correlation of TMA level with eating quality (15); at a given level of TMA, fish caught in the summer and late fall usually showed more rapid organoleptic deterioration than those caught in the spring and winter (16).

Excellent reviews on methodology for TMA determination are given by Shewan et al. (17) and Hebard et al. (3). Although subject to interference and occassional error, the picric acid method of Dyer (18, 19, 1) modified by Tozawa and co-workers (20) has been the most widely used and evaluated (3) and has been adapted by the Association of Analytical Chemists (21). The method as originally reported was subject to interference by amines particularly DMA if the samples were drawn from frozen storage (22, 23) or if the samples were in advanced stages of decomposition (22, 19, 24). The interference of DMA was used to advantage by Castell and co-workers (25) who applied the picric acid method with either 25% KOH or 50% K_2CO_3 as TMA liberating agents. By comparing values obtained by the two bases, a simultaneous equation could be solved producing a corrected TMA and DMA value. Researchers are now turning to more automated methods (HPLC, especially) for more accurate determination of TMA (26).

a. Application

The picric acid method is applicable to all marine species containing the precursor TMAO for the estimation of bacterial spoilage. However, as noted in the discussion preceeding, TMA correlation with organoleptic quality or grades will vary with species, season and stock. A survey of TMA values from the literature has been compiled by Hebard et al. (3). When the sample has been stored frozen for extended periods of time, the simultaneous TMA/DMA procedure should be applied as described in method 12 to overcome DMA interference.

b. Principle

A trichloroacetic acid (or 6% perchloric acid) extract is obtained from the fish and reacted with picric acid to produce a highly colored picrate. Formaldehyde is added in the analysis to suppress reaction with ammonia and monomethylamine. Addition of an alkali (25% KOH) facilitates extraction of the amine to the toluene phase with subsequent formation of the picrate. By measuring absorbance at 410 nm, TMA may be quantitated.

c. Precautions

1. Adequate shaking/rotation is important. Deviation in procedure may alter the standard curve.

2. Temperature during mixing and shaking of solutions of TCA extract, formaldehyde, alkali, and toluene should be maintained at 30°C to ensure reproducibility (20). A water bath at this temperature should be at hand.

3. Only dry a small amount of picric acid for use. Picric acid will detonate if subjected to shock or heat when in a dry state. Stock bottle should be kept moist with distilled water. Picric acid should never be dried in an oven.

4. Dry toluene over Na_2SO_4 or molecular seives.

5. Picric acid reagent should be added to clean dry test tubes since the method is sensitive to water and other contaminants.

6. If the sample has been stored in frozen storage for extended periods or at a temperature accelerating production of DMA, the simultaneous DMA/TMA procedure outlined in laboratory method 12 should be applied to compensate for DMA interference.

7. Maintain samples as cold as possible prior to analysis to minimize further TMA production.

8. TCA extracts are stable in frozen storage.

9. If perchloric acid (6%) is used for extraction extracts must be carefully neutralzed to pH 6.8 just before use. Exercise caution in use of perchloric acid.

SAMPLE PREPARATION

Select representative samples for analysis. Since TMA increases with bacterial levels, if variation in pseudomonad numbers is expected in different areas of the same pack (top vs bottom for example), sampling should reflect this.

Comminute fillets in a food processor and weigh portions (50 g) into portion cups. Samples after portioning may be stored for a short time (few days) at -35°C without detriment. Storage at room temperature or at refrigerator temperatures should be avoided since TMA production will continue under these conditions.

APPARATUS

1. **Blender** with small blender jar

2. **Low speed centrifuge** optional

3. **Rotator,** eg. Cole-Palmer Model 7621-20

4. **Spectrophotometer** for use at 410 nm

5. **Filter paper** Whatman #4 or glass wool

6. **Glassware:** Funnels, 50 ml Erlenmeyer flasks, screw capped (Teflon lined) culture tubes (20 x 150 mm)

REAGENTS

1. **Stock TMA Standard (1.00 mg TMA-N/ml):** Dissolve 0.6820 g TMA-HCl weighed to nearest 0.0001 g (dried overnight in desiccator) in distilled water. Dilute to 100 ml. Store refrigerated.

2. **Working TMA Standard (10.0 µg TMA-N/ml):** Pipet 1 ml TMA stock solution into a 100 ml volumetric flask and dilute to volume.

3. **Formaldehyde (10%):** Dilute 26.8 ml formalin (37.3% HCHO) to 100 ml with distilled water.

4. **KOH (25% w/w):** Carefully and with stirring dissolve 25g KOH in 75 ml distilled water. Cool.

5. **Trichloroacetic acid (7.5%):** Dissolve 7.5 g TCA in 92.5 ml distilled water.

6. **Moisture free toluene:** Dry over anhydrous Na_2SO_4. Not necessary to distill.

7. **Sodium sulfate,** granular anhydrous

8. **Stock picric acid reagent:** Carefully dissolve 2.0 g picric acid (dried overnight in desiccator at room temperature - DO NOT USE DRYING OVEN) in moisture-free toluene. Dilute to 100 ml

with dried toluene.

N.B. Picric acid should be kept moist at all times to prevent explosion. When preparing standard only a small amount should be dried at room temperature; handle with caution.

9. **Working picric acid reagent:** Pipet 1 ml picric acid stock reagent (with bulb) to a 100 ml volumetric flask and dilute to volume with dried toluene.

PROCEDURE

A. Preparation of Extracts

1. Add 50 g sample and 100 ml 7.5% TCA (1:2 extraction) to a small blender jar and blend thoroughly.

2. Centrifuge at 4°C for 15 minutes at about 2000 x g (ca 4000 rpm). An alternate purification procedure to centrifugation and decanting is filtration through Whatman #4 filter paper.

3. Decant supernatant fluid through glass wool.

4. Extracts may be frozen at -20°C for extended periods with little deterioration.

B. Analysis of TCA Extracts

1. Pipet aliquots (0.1 to 4 ml) of extract into 20 x 150 mm screw capped tubes. Concentration of TMA in extract will determine appropriate aliquot size.

2. For standard curve, use 1.0, 1.5, 2.5 and 3.0 ml of **working** standard solution.

3. Add water to each tube to bring total volume to 4.0 ml for both extracts and standards. For a blank use 4.0 ml distilled water.

4. To each tube add in progressive order 1 ml 10% formaldehyde, 10 ml toluene and, 3.0 ml 25% KOH. Ensure temperature is 30°C (see precautions note #2).

5. Mix for 15 min on rotator. Efficient mixing is important.

6. With pasteur pipette, remove ca 7 ml of upper toluene layer from each tube to a large drying tube containing approximately 0.3 to 0.4 g anhydrous Na_2SO_4. Shake gently (vortex mixer) until the solutions are clear.

7. Pipet 5 ml of picric acid **working** reagent into clean dry test tubes. If color change (yellow) is evident when reagent is added to empty tubes, contamination is present and clean dry test tubes should be obtained.

8. Pipet 5 ml of toluene solution (from Step 6) into tubes containing picric acid reagent and mix by swirling gently.

9. Measure absorbance at 410 nm.

CALCULATIONS

1. For standard curve, plot absorbance of toluene solutions vs. µg TMA-N added to test tube as shown in Figure 9.1 below:

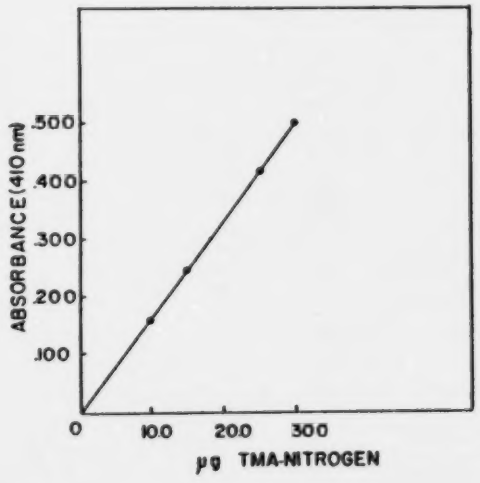


Figure 9.1 Typical TMA standard curve

2. For each absorbance (sample) determine the equivalent µg TMA-nitrogen from the graph.

3. Calculate TMA content from the formula:

TMA =

$$\frac{\text{TMA in tube x total vol. extractant phase}}{\text{vol. added to test tube x wt. fish extracted}}$$

$$= \frac{T \times [V_1 + (0.01 \times M \times W)]}{V_2 \times W \times 10} \qquad (1)$$

Where:

TMA = trimethylamine expressed as mg TMA-N per 100g fish

T = equivalent TMA in µg determined from standard curve

M = moisture of fish sample expressed in percent

V_1 = volume (ml) of TCA added for 1:2 extraction

V_2 = volume (ml) of extract added to test tube

W = weight (g) of fish used in 1:2 extraction

EXAMPLE

Cod extract was produced by adding 50 g (W) of cod at 80% (M) moisture to 100 ml (V_1) of 7.5% TCA, blending, and filtering. A 2 ml (V_2) sample was used for analysis.

From the standard curve, the equivalent TMA-N in the test tube was 22.0 µg (T).

The TMA content of the fish may be calculated from formula (1) as:

$$\text{TMA} = \frac{22.0 \times [100 + (0.01 \times 80 \times 50)]}{2 \times 50 \times 10}$$

$$= \frac{22.0 \times 140}{1000}$$

= 3.08 mg TMA-N per 100 g

REFERENCES

1. LOVE, R.M. 1970. The Chemical biology of fishes. Academic Press, New York.

2. REGENSTEIN, J.M., M.A. Scholsser, A. Samson, and M. Fey. 1982. Chemical changes of trimethylamine oxide during fresh and frozen storage of fish. In Chemistry and Biochemistry of Marine Food Products (R.E. Martin, G.J. Flick, C.E. Hebard, and D.R. Ward, eds.) AVI Pub. Conn. pg 137-148.

3. HEBARD, C.E., G.J. Flick, and R.E. Martin. 1982. Occurrence and significance of trimethylamine oxide and its derivatives in fish and shellfish. In Chemistry and Biochemistry of Marine Food Products (R.E. Martin, G.J. Flick, C.E. Hebard, and D.R. Ward, eds.) AVI Pub. Conn. pg 149.

4. HARADA. 1975. Studies on enzymes forming formaldehyde and dimethylamine in fish and shellfish. J. Shimonosaki Univ. Fisheries 23:163.

5. LAYCOCK, R.A. and L.W. Regier. 1971. Trimethylamine producing bacteria on haddock (Melanogrammus aeglefinus) fillets during refrigerated storage. J. Fish. Res. Bd. Can. 28:305.

6. CASTELL, C.H., M.F. Greenough, R.S. Rodgers, and A.S. Macfarland. 1958. Grading fish for quality. I. Trimethylamine values of fillets cut from graded fish. J. Fish. Res. Bd. Canada 15:701.

7. SHEWAN, J.M., and N.R. Jones. 1957. Chemical changes occurring in cod muscle during chill storage and their possible use as objective indices of quality. J. Sci. Food. Agric. 8:491.

8. CASTELL, C.H., and M.F. Greenough. 1958. Grading fish for quality. Grading of recently cut fillets. J. Fish. Res. Bd. Canada 15:729.

9. HOOGLAND, P.L. 1958. Grading fish for quality. 2. Statistical analysis of results of experiments regarding grades and trimethylamine values. J. Fish. Res. Bd. Canada 15:717.

10. LUIJPEN, A.F.M.G. 1958. Objective spoilage tests for fish stored under conditions other than normal chilling in ice. J. Sci. Food Agric. 9:410.

11. CASTELL, C.H., M.F. Elson and J.G. Giles. 1961. Grading fish for quality 4. Variations in the relation between trimethylamine values and grades for gutted, trawler caught Atlantic cod and haddock. J. Fish. Res. Bd. Can. 18:303.

12. HILLIG, F., L.R. Shelton, Jr., and J.H. Loughrey. 1962. Summary of chemical data on progressive decomposition studies of cod, haddock, and perch. J. Assoc. Off. Anal. Chem. 45:724.

13. KOVAL'CHUK, G.K., and N.F. Moskalenko. 1961. Volatile alkaline matter and trimethylamine in meat of various fishes caught in Azov-Black Sea Basin. Rybn. Khoz. 37:64. Chem. Abstr. 56, 15894E, 1962.

14. SIGURDSON, G.J. 1947. Comparison of chemical tests of the quality of fish. Anal. Chem. 19:892.

15. BURT, J.R., D.M. Gibson, A.G. Jason, and H.R. Sanders. 1976. Comparison of methods of freshness assessment of wet fish. II. Instrumental and chemical assessments of boxed experimental fish. J. Food Technol. 11:73.

16. CASTELL, C.H., and J.G. Giles. 1961. Spoilage of fish in the vessels at sea. 7. Further studies on seasonal variations in the landed quality of gutted, trawler-caught Atlantic cod and haddock. J. Fish. Res. Bd. Canada 18:295.

17. SHEWAN, J.M., D.M. Gibson, and C.K. Murray. 1971. The estimation of trimethylamine in fish muscle. In Fish Inspection and Quality Control. R. Kreuzer (ed). Fishing News (Books) Ltd. London pp 183-186.

18. DYER, W.J., and Y. A. Mounsey. 1945. Amines in fish muscle II. Development of trimethylamine and other amines. J. Fish. Res. Bd. Canada 6:359.

19. DYER, W.J. 1959. Report on Trimethylamine in fish. J. AOAC 42:292.

20. TOZAWA, H., K. Enokiharo, and K. Amano. 1971. Proposed modification of Dyer's methods for trimethylamine determination in cod fish. In Fish Inspection and Quality Control. R. Kreuzer (ed) Fishing News (Books) Ltd. London p 187-190.

21. "Official and Tentative Methods of the American Oil Chemists' Society", Vol. 1, Third Edition, AOCS, Champaign, IL, 1971.

22. CASTELL, C.H. 1971. Metal catalyzed lipid oxidation and changes of proteins in fish. J. Am. Oil Chem. Soc. 48:645.

23. TOKUNAGA, T. 1964. Studies on the development of dimethylamine and formaldehyde in Alaska pollock muscle during frozen storage. Bull. Hokkaido Reg. Fish. Res. Lab. 29:108.

24. BULLARD, F. A. and J.Collins, 1980. An improved method to analyze trimethylamine in fish and the interference of ammonia and dimethylamine. Fishery Bulletin 78:465.

25. CASTELL, C.H., B. Smith, and W.J. Dyer. 1974. Simultaneous measurements of trimethylamine and dimethylamine in fish, and their use for estimating quality of frozen stored gadoid fillets. J. Fish. Res. Bd. Canada 31:383.

26. GILL, T.A., and J. W. Thompson. 1984. Rapid, automated analysis of amines in seafood by ion-moderated partition HPLC. J. Food Science. 49:603.

10. TOTAL VOLATILE BASE (TVB-1 and TVB-2)

GENERAL DISCUSSION

Microbiological activity and biochemical changes during iced or chilled storage of fish are responsible for the progressive decline of organoleptic quality. Specifically, enzymatic (bacterial and natural) breakdown of both proteins and the osmoregulatory agent trimethylamineoxide (TMAO) present in marine species (1) result in a build-up of odoriforous compounds, namely: ammonia, monomethylamine, trimethylamine and other volatile amines (2, 3, 4) - see General Discussion section, laboratory method 9, TMA content.

Total volatile base (TVB) analyses measuring low molecular weight volatile bases and amine compounds produced by microbial decarboxylation of amino acids (5, 6) have been commonly used for assessment of fresh fish quality (7, 8). In fact, more data have probably been accumulated on this test than for any other spoilage indicator (7). Of 65 papers attempting to correlate TVB with organoleptic quality reviewed by Borgstrom (7), 39 were positive, 17 were negative and 9 found variable results. However excellent linear correlation between TVB and TMA values has been reported for cod fillets (9). Recently, Botta and co-workers (10, 11) applied six different published methods to Atlantic cod stored on ice for up to 18 days and found that although TVB nitrogen increased with time for all procedures, values differed from each other substantially.

The TVB test involves distillation of amine compounds into boric acid solution and titration with a standardized acid (HCl or H_2SO_4). Samples have been in the form of press juice (12, 13), comminuted tissue (14) and 60% ethanol (12), 5-20% TCA (15, 16) perchloric acid (17), or magnesium sulfate (8) extracts. Techniques for amine to boric acid transfer have included microdiffusion (18, 19, 15, 16, 12), atmospheric distillation (14, 12, 20), vacuum distillation (8), and aeration (21, 22, 12). With this choice in methodology, literature values for organoleptic acceptability limits have ranged from 20 to 30 mg TVB nitrogen per 100 grams fish. Additional discrepancies in data have also arisen among laboratories due to the varied nature of heating apparatus in use. These factors coupled with species differences are responsible for the range of reported values (7) and the risk involved in making direct comparisons.

Two TVB procedures are presented, i.e. by direct magnesium oxide (method A) and magnesium sulfate extract (Method B) analysis. While there is good correlation for data from the two procedures for cod and squid (23) the former method would be expected to yield higher TVB values than the latter for species where no published comparison exists (11). Since the magnesium sulfate procedure utilizes an extract, TVB values obtained would depend on efficiency of extraction which could affect correlation with the direct method for certain species. This method also utilizes large quantities of magnesium sulfate. On the other hand the extraction method would be less prone to interference from protein degradation during distillation. Whichever method is chosen, a "baseline" value should be experimentally established with good quality fish. Figure 10.1 below illustrates an approximate correlation on TVB values from the two procedures for cod and squid.

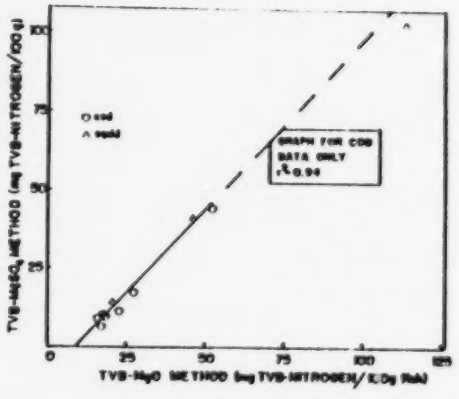


Figure 10.1 Correlation of TVB (MgO vs $MgSO_4$) methods for cod and squid.

10. TOTAL VOLATILE BASE

a. Application

The total volatile base (TVB) determination is applicable to the estimation of enzymatic and bacterial (Alteromonad) spoilage in marine fish. Although some controversy exists regarding correlation of TVB with organoleptic quality (7), TVB may be correlated with TMA for cod fillets stored at +2°C (9) but will vary with species and laboratory procedure (10, 11).

Of the two TVB methods presented, the first or magnesium oxide method (A) is suggested for "first action" analysis. Baseline values for good quality fish and if possible cut-off values should be established on available "in-house" laboratory equipment.

b. Principle

Volatile bases (ammonia, monomethylamine, dimethylamine, trimethylamine, and other volatile amines) are distilled into boric acid from an extract (or solution containing blended sample) after being rendered basic by the addition of magnesium oxide or sodium hydroxide. The boric acid is titrated to its original state with a strong acid (at low concentration) to determine the amount of base distilled.

c. Precautions

1. Do not store samples for extended periods at room temperature or under refrigeration since microbiological activity will raise TVB levels. Store frozen.

2. Heat at maximum attainable with heating mantles.

3. Match size of mantles to flasks; use of oversized mantles will result in their deterioration due to overheating.

4. Distillation times and volumes as prescribed must be strictly adhered to.

5. Accurately standardize pH meter (Method 8) with pH 1 and 3 buffers. Readings at such low pH may sometimes drift depending on meter and condition of electrode. Extra time may also be necessary for equilibration (see laboratory method 1, pH measurement).

SAMPLE PREPARATION

Thoroughly comminute a representative sample in a food processor and preweigh 10 g portions for TVB-1 or 30 g portions for TVB-2. Samples must be stored refrigerated or on ice (short time only) to retard further microbiological production of volatile bases. If portions are to be held more than two hours they should be frozen at -30°C or colder where they may be stored for up to 2 weeks with little change.

A. TOTAL VOLATILE BASE 1 (TVB-1)-direct MgO method (recommended "first action)

APPARATUS (TVB-1)

1. **Vertical distillation apparatus** as illustrated in Figure 10.2
2. **Heating mantle**
3. **Waring blender** or Polytron (Brinkmann Instruments Ltd.)
4. **Drying oven** at 103°C
5. **Glassware:** 500 ml Erlenmeyer flask, three 125 ml Erlenmeyer flasks, 1000 ml round bottom flask, 50 ml buret, weighing bottle, watch glass

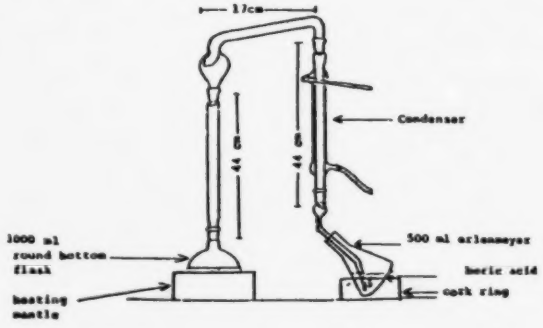


Figure 10.2 Vertical distillation apparatus for TVB (from Woyewoda and Ke [20])

1. **Boric acid solution (2%):** Dissolve 20 g boric acid in 1 liter distilled water.
2. **Sodium Hydroxide (0.1N):** Dissolve 4 g NaOH in small volume of distilled water. Make up to 1 liter with distilled water. Standardize according to method A below.
3. **Sulphuric acid (0.050 N):** To 1 liter distilled water add 1.39 ml concentrated H_2SO_4. Standardize against standard NaOH solution (according to method B below).
4. **Screened methyl red indicator:** To 100 ml ethanol add 0.05 g methyl red and 0.075 g bromocresol green.
5. **Magnesium oxide**
6. **Anti-bumping granules,** BDH Chemicals Ltd.
7. **Anti-foam silicon preparation**
8. **Potassium acid phthalate,** analytical grade 3 g dried overnight at 103°C.
9. **Phenolphthalein indicator:** dissolve 1 g phenolphthalein in 100 ml ethanol (95%).

PROCEDURE (TVB-1)

A. Standardization of 0.1N NaOH

1. Into each of three clean 250 ml Erlenmeyer flasks, weigh 0.30 g of dried potassium acid phthalate. Add 50 ml of distilled water (to dissolve) and 3 drops phenolphthalein indicator to each flask.
2. Fill a 50 ml buret with sodium hydroxide solution (ca 0.1N).
3. Titrate each potassium acid phthalate sample to the endpoint of the phenolphthalein indicator (first perceptible but permanent pink color) with 0.1N NaOH.
4. Record the position of the meniscus on the buret and the volume of NaOH used.
5. Calculate NaOH normality as:

$$N_1 = \frac{W_1 \times 1000}{204.22 \times V_1} \qquad (1)$$

where: N_1 = normality NaOH
V_1 = volume (ml) NaOH used for titration
W_1 = weight (g) potassium acid phthalate in flask

6. Repeat for each of the remaining phthalate samples and calculate average normality of the NaOH.

B. Standardization of 0.05N H_2SO_4

1. Pipette 25 ml aliquots of prepared H_2SO_4 solution into each of 3 clean 125 ml Erlenmeyer flasks and 3 drops of phenolphthalein indicator.

2. Titrate each to the phenolphthalein endpoint (first perceptible but permanent (30 sec) pink color) with standardized 0.1N NaOH (50 ml buret).

3. Record the volume of NaOH used for the titration.
Repeat for each of the three samples. Calculate the average volume of NaOH used; results should be within 0.03 ml. Calculate H_2SO_4 normality as:

$$N_2 = \frac{N_1 \times V_2}{V_3} \qquad (2)$$

where: N_1 = normality NaOH
N_2 = normality H_2SO_4
V_2 = average volume (ml) NaOH used for titrations
V_3 = volume (ml) H_2SO_4 added to each Erlenmeyer

C. Determination of TVB-1

1. To a 1000 ml round bottom distillation flask, add 10 g sample and 300 ml distilled water. Blend by inserting Polytron probe into the flask (alternately use blender).

2. Then add anti-bumping granules and 2 g magnesium oxide, swirl.

3. Connect to still.

4. To 250 ml Erlenmeyer receiving flask, add 25 ml of 2% boric acid and a few drops of indicator.

5. Install receiving flask such that the receiver tube dips below the boric acid solution (see Figure 10.2).

6. Heat the distilling flask so that liquid boils in exactly 10 minutes.

7. Using the same rate of heating, distill for exactly 25 minutes.

8. After distillation, titrate the solution in the receiver flask back to the original color, using standard 0.05N H_2SO_4 solution.

9. Titrate a blank which has all reagents except the sample. If the blank requires more than 0.1 ml titrant, new boric acid should be used.

CALCULATION (TVB-1)

Total volatile bases expressed as milligrams nitrogen per 100 g samples:

$$TVB = \frac{(V_4 - V_5) \times N_2 \times 100 \times 14}{W_2} \qquad (3)$$

Where:

V_4 = volume (ml) H_2SO_4 used for sample
V_5 = volume (ml) H_2SO_4 used for blank
N_2 = Normality of H_2SO_4
W_2 = weight of sample in grams

EXAMPLE (TVB-1)

Squid, 10 g (W_2), was analyzed for TVB. In the titration, 2.75 ml (V_4) of 0.053 N H_2SO_4 (N_2) was used. Blank was 0.02 ml (V_5). Total volatile base from formula 3 is:

10. TOTAL VOLATILE BASE

$$TVB = \frac{(2.75 - 0.02) \times 0.053 \times 100 \times 14}{10}$$

$$= \frac{2.73 \times 0.053 \times 100 \times 14}{10}$$

= 20.2 mg N/100 g

= 20.2 mg-N%

B. TOTAL VOLATILE BASE (TVB-2) - $MgSO_4$ extraction method (recommended alternate method)

APPARATUS (TVB-2)

1. **Distillation apparatus:** micro-Kjeldahl steam distillation (Figure 4.1), macro-Kjeldahl distillation unit or vertical distillation unit (Figure 10.2)

2. **Blender**

3. **Filter paper** Whatman #1

4. **Glassware:** Buchner funnel, weighing bottle, watch glass, 250 ml Erlenmeyer flasks (3), 50 ml buret, 125 ml Erlenmeyer flasks (3)

5. **Drying oven** at 103°C

6. **pH meter,** with combination pH electrode

REAGENTS (TVB-2)

1. **Magnesium sulfate solution:** 600 g of $MgSO_4$ $7H_2O$ dissolved in distilled water. Add 20 ml of 6N H_2SO_4 (or 3.5 ml concentrated H_2SO_4) and dilute the solution to 1000 ml with distilled water.

2. **Sodium hydroxide (20% w/v):** Dissolve 20g NaOH in 100 ml distilled water.

3. **Sodium hydroxide (0.1N):** In a 1000 ml volumetric flask, dissolve 4 g NaOH in a small volume of distilled water. Make up to 1 liter with distilled water.

4. **Potassium acid phthalate,** analytical grade, 3 g dried overnight at 103°C.

5. **Phenolphthalein indicator:** dissolve 1 g phenolphthalein in 100 ml ethanol (95%).

6. **Hydrochloric acid (0.1N):** In one litre distilled water, dissolve 8.3 ml concentrated HCl.

7. **Screened methyl red indicator:** to 100 ml ethanol add 0.05 g methyl red and 0.075 g bromocresol green.

8. **Boric acid solution (2% w/v),** dissolve 20 g boric acid in 1 liter of distilled water.

9. **Sulphuric Acid (1N):** to 75 ml distilled water add 2.8 ml concentrated H_2SO_4, mix and dilute to 100 ml.

10. **Sodium Hydroxide (1N):** dissolve 4 g NaOH in a small volume of distilled water and dilute to 100 ml.

PROCEDURE (TVB-2)

A. Standardization of 0.1 N NaOH

1. Into each of three clean 250 ml Erlenmeyer flasks, weigh 0.30 g of dried potassium acid phthalate. Add 50 ml of distilled water (to dissolve) and 3 drops phenolphthalein indicator to each flask.

2. Fill a 50 ml buret with sodium hydroxide solution (ca 0.1N).

3. Titrate each potassium acid phthalate sample to the endpoint of the phenolphthalein indicator (first perceptible but permanent pink color) with 0.1N NaOH.

4. Record the position of the meniscus on the buret and the volume of NaOH used.

5. Calculate NaOH normality as:

$$N_1 = \frac{W_1 \times 1000}{204.22 \times V_1} \qquad (1)$$

where: N_1 = normality NaOH
V_1 = volume (ml) NaOH used for titration
W_1 = weight (g) potassium acid phthalate in flask

6. Repeat for each of the remaining phthalate samples and calculate average normality of the NaOH.

B. Standardization of 0.1N HCl

1. Pipette 25 ml aliquots of prepared HCl solution into each of 3 clean 125 ml Erlenmeyer flasks and 3 drops of phenolphthalein indicator.

2. Titrate each to the phenolphthalein endpoint (first perceptible but permanent (30 sec) pink color) with standardized 0.1N NaOH (50 ml buret).

3. Record the volume of NaOH used for the titration.
Repeat for each of the three samples.
Calculate the average volume of NaOH used; results should be within 0.03 ml.
Calculate HCl normality as:

$$N_3 = \frac{N_1 \times V_6}{V_7} \qquad (4)$$

where: N_1 = normality NaOH
N_3 = normality HCl
V_6 = average volume (ml) NaOH used for titrations
V_7 = volume (ml) HCl added to each Erlenmeyer

C. Determination of TVB-2

1. Blend 30 g sample with 70 ml distilled water for one minute.

2. Add 100 ml $MgSO_4$ solution and blend again for one minute.

3. Add additional 100 ml $MgSO_4$ solution and re-blend for one minute. Allow to stand a few minutes to ensure separation of foam from liquid.

4. Filter through Buchner funnel, using Whatman #1 paper.

5. Adjust filtrate to pH 2 (with 1N H_2SO_4 or 1N NaOH). Ensure pH meter is accurately standardized at pH 1 and 3. Check linearity by measuring pH of standard pH 2 buffer. Extra time may be required for equilibration of readings (see laboratory method 1, measurement of pH).

6. Add 25 ml sample extract to distillation assembly:

   a. **With macro-Kjeldahl unit**

      i) Rinse sample in with 200 ml distilled water (Silicone defoamer may be used to control foaming), add 5 ml of 20% NaOH, then quickly rinse neck of flask with distilled water from wash bottle.

      ii) Quickly assemble distillation unit. Place receiving flask containing 20 ml of boric acid such that the outlet of the still is below the level of boric acid.

      iii) Begin heating immediately. Collect 100 ml of distillate.

   b. **With vertical distillation unit** (see Figure 10.2), use same procedure as described for macro-Kjeldahl unit.

   c. **With micro-Kjeldahl unit,** (see Figure 4.1)

      i) Rinse sample into still with a small amount of distilled water from a wash bottle.

      ii) Add 5 ml of 20% NaOH, wash in quickly with distilled water and immediately close stopcock.

      iii) Place receiving flask containing 20 ml of boric acid such that the outlet of the still is below the level of boric acid.

      iv) Turn on the steam supply and collect 50 ml of distillate.

7. Add 5 drops of screened methyl red indicator to distillate.

8. Titrate with standard 0.1 N HCl (standard 0.01 N HCl may be used to better control the endpoint and to increase the volume; prepare by 10 fold dilution of standard 0.1N HCl).

9. A blank, containing all reagents except the sample, should also be distilled and titrated.

CALCULATIONS (TVB-2)

Total volatile bases expressed as mg TVB-N/100 g sample:

$$TVB = \frac{(V_8 - V_9) \times N_3 \times 1400 \times f}{W_3} \qquad (5)$$

Where:

TVB = mg total volatile base nitrogen per 100 g sample
V_8 = ml HCl to titrate samples
V_9 = ml HCl to titrate blank
N_3 = normality of HCl titrant
W_3 = sample wt (g)
f = dilution factor (300/25)

REFERENCES

1. LOVE, R.M. 1970. The Chemical Biology of Fishes. Academic Press, New York.

2. REGENSTEIN, J.M., M.A. Scholsser, A. Samson, and M. Fey. 1982. Chemical changes of trimethylamine oxide during fresh and frozen storage of fish. In Chemistry and Biochemistry of Marine Food Products (R.E. Martin, G.J. Flick, C.E. Hebard, and D.R. Ward, eds.) AVI Pub. Conn. pg 137-148.

3. LAYCOCK, R.A. and L.W. Regier. 1971. Trimethylamine producing bacteria on haddock (Melanogramm aeglefinus) fillets during refrigerated storage. J. Fish. Res. Bd. Can. 28:305.

4. HEBARD, C.E., G.J. Flick, and R.E. Martin. 1982. Occurrence and significance of trimethylamine oxide and its derivatives in fish and shellfish. In Chemistry and Biochemistry of Marine Food Products (R.E. Martin, G.J. Flick, C.E. Hebard, and D.R. Ward, eds.) AVI Pub. Conn. pg 149.

5. JACOBEN, L.F. and A.G. Rand, Jr. 1982. Biochemical Evaluation of seafood. In Chemistry and Biochemistry of Marine Food Products (eds. R.E. Martin, G.J. Flick, C.E. Hebard and D.R. Ward) AVI Pub., Conn. pp 347-365.

6. ESKIN, N.A., H.M. Henderson, and R.J. Townsen, 1971. Biochemistry of Foods. Academic Press, New York.

7. FARBER, L. 1965. Freshness Tests. In Fish as Food (ed. G. Borgstrom). Academic Press, New York.

8. DACOSTA, A.A., T. Tomizama, and J.A. Stern. 1956. The applicability of the vacuum distillation technique for the determination of volatile acids and volatile bases in fish flesh. In Chilling of Fish. Fish Processing Technologists Meeting (ed. Netherlands, Ministry of Agriculture, Fisheries and Food) pub. 1960. pp 244-250.

9. WOYEWODA, A.D., D.S. Singer, and E.G. Bligh. 1982. Estimation of TMA in fresh or frozen cod and squid by the ammonia electrode. 27th Atlantic Fisheries Technological Conf. Sept, 1982, Portland, Me.

10. BOTTA, J.R., J.R. Lauder, and M.A. Jewer. 1982. Total Volatile Basic Nitrogen (TVB-N) as an index of quality of fresh fish. 27th Atlantic Fisheries Technological Conference, Sept., 1982. Portland, Maine.

11. BOTTA, J. R., J. T. Lauder, and M. A. Jewer, 1984. Effect of methodology on total volatile basic nitrogen (TVB-N) determination as an index of quality of fresh Atlantic Cod (Gadus morhua) J. Food Science 49:734.

12. STANSBY, M.E., R.W. Harrison, J. Dassow, and M. Sater. 1944. Determining volatile bases in fish. Comparison of precision of certain methods. Ind. and Eng. Chem. 16:593.

13. BEATTY, S.A. and N.E. Gibbons. 1937. The measurement of spoilage in fish. J. Biol. Bd. Can. 3:77.

14. COX, H.E., and D. Pearson. 1962. In Chemical Analysis of Foods. Chemical Publishing Co. Inc., N. York.

15. MONTGOMERY, W.A. 1956. A rapid method for the estimation of volatile bases in fish muscle. In chilling of Fish. Fish Processing Technologists Meeting (ed. Netherlands Ministry of Agriculture, Fisheries and Food) Pub. 1960. pp 234-242.

16. NAIR, R.B., P.K. Tharamani, and N.L. Lahiry. 1971. Studies on chilled storage of fresh water fish during iced storage. J. Fd. Sci. Techn. (India) 8:53.

17. THOMSON, A.B., H.K. Davis, J.C. Early and J.R. Burt. 1974. Spoilage and spoilage indicators in queen scallops (Chlamys operularis). I. Held in ice. J. Food Technol. 9:381-390.

18. CONWAY, E.J. 1947. Microdiffusion analysis. Rev. Ed., D. VanNostrand Co., Inc., New York.

19. MOORJANI, M.N., J.R. Iyengar, K. Visweswariah, D.S. Bhatia, and V. Subrahmanyan. 1958. Food Technology 12:305.

20. WOYEWODA, A.D. and P.J. Ke. 1980. Laboratory Quality Assessment of Canadian Atlantic Squid (Illex illecebrosus)Department of Fisheries and Oceans, Halifax, Technical Report No. 902.

21. KAWABATA, T. 1953. Fundamental studies on the determination of volatile basic nitrogen by aeration method. III. Special factors affecting the velocity constant in removing the volatile base. Bull. J. Soc. Sci. Fish. 18:525.

22. TOMIYAMA, T.B. 1952. Bull. J. Soc. Sci. Fish. 17:405.

23. WOYEWODA, A. D, and S. J. Shaw. Unpublished data.

11. HYPOXANTHINE

GENERAL DISCUSSION

Fish spoilage occurs at two levels: microbial and autolytic (1). After death both processes which are closely controlled during life, are limited only by environmental factors and the availability of suitable raw material. Bacterial numbers and levels of decomposition products have been used as indicators of post mortem quality. However, the measurement of nucleotides such as hypoxanthine (Hx) for use in fish quality assessments provides several distinct advantages over other objective chemical tests such as trimethylamine (TMA), dimethylamine (DMA) and total volatile bases (TVB) and others which essentially measure bacterial spoilage (2). The accumulation of Hx in fish tissue reflects the initial phases of autotylic deterioration (3-7) as well as later contributions through bacterial spoilage. Hx concentrations are not affected by heat processing (8) or irradiation (9) and are very useful in certain freshwater fish which contain little or no trimethylamine oxide (TMAO) (10) which renders the TMA analysis useless.

A decrease in ATP levels triggers the onset of rigor mortis and the accompanying nucleotide degradation (11, 4, 5, 1, 12, 13, 14, 15, 16, 2). The biochemical reaction involving Hx production follows the sequence (17):

$$\underset{\text{(a)}}{\text{ATP}} \xrightarrow{\text{(b)}} \text{ADP} \xrightarrow{\text{(c)}} \text{AMP} \xrightarrow{\text{(d)}} \text{IMP} \xrightarrow{\text{(e)}} \text{INO} \xrightarrow{\text{(f)}} \text{Hx} \xrightarrow{\text{(g)}} \text{X} \longrightarrow \text{U}$$

where ATP is adenosine-5'-triphosphate, ADP is adenosine-5'-diphosphate, AMP is adenosine-5'-monophosphate, IMP is inosine-5'-monophosphate, INO is inosine, X is xanthine, and U is uric acid. In most species, the rate determining step is (e) or (f) therefore INO and Hx accumulate with time (18).

Hx concentrations peak and then decrease with progressive spoilage. Average maximum Hx levels in seafood were reported as 5 μM/g with a range of 3.0 - 7.5 μM/g (19, 14, 20, 21). However actual levels observed have been found to be dependent on genus, sampling procedures, seasonal differences and environmental factors (22).

One of the most popular methods for monitoring Hx levels in fish tissue is the enzymatic method recommended by the Analytical Methods Committee (23). This is essentially the same method as described by Jones et al (4) where xanthine oxidase (XO) is used to rapidly convert Hx to X and subsequently to U. Modifications to the method have led to automation by making use of a redox indicator dye (24) test paper strips (13, 25) and colorimetric enzyme assay procedures. An enzyme sensor has been developed for routine determinations of Hx (18, 26).

Hx has been isolated by ion-exchange column chromatography (27, 28) and TLC however, recently a rapid and specific liquid chromatographic (LC) method for determining Hx content in fish tissues has been developed (30). Hx is extracted with 0.6 M perchloric acid and determined on a reversed phase column with UV absorbance detection.

Two procedures are presented, the enzymatic method of Jones et al (4) and the LC method of Burns and Ke (30). There is good correlation of data from the two procedures especially with cod. The LC method has been proven to have several advantages over the XO method such as measurement of Hx directly rather than relying on its conversion to U, the ability to remove interfering compounds chromatographically, increased sensitivity, and measurement of other nucleotides. However, whichever method is chosen for the measurement of Hx, baseline values must be experimentally established for each type of fish. Hx concentrations have already proven to correlate very well with eating quality in a number of fish species (4, 31, 9, 12, 30).

a. Application

The hypoxanthine test is applicable to all species of fish in early stages of refrigerated deterioration. The analysis should be combined with another analysis such as TMA since hypoxanthine levels decline after attaining a maximum. Species variation can be expected as already mentioned.

b. **Principle**

1. Enzymatic Method

The analysis (4) utilizes a perchloric acid extract of the fish tissue. Through the action of the added enzyme xanthine oxidase, hypoxanthine in the presence of oxygen is converted to xanthine and then uric acid which may be quantitated spectrophotometrically at 290 nm. The procedure incorporates a number of "blank" corrections.

2. LC Method

Hx and other nucleotides are extracted from fish tissue utilizing a perchloric acid extract. Hx is determined in the neutralized extract by LC on a reversed phase column with UV absorbance detection at 254 nm.

c. **Precautions**

1. Since these tests measure early stages of spoilage the sample flesh should be excised and blended with the extracting acid medium as quickly as possible. Unextracted samples should be kept cold to retard enzymatic activity.

2. Caution must be exercised when working with perchloric acid. According to the Merck Index, perchloric acid combines vigorously with water with evolution of heat, is extremely caustic and can burst into flames when in contact with oxidizable matter (paper, wood, etc.)

3. Extracts may be stored frozen after neutralization without undue loss of hypoxanthine.

SAMPLE PREPARATION

Comminute a representative sample of fish flesh in a Cuisinart food processor taking care to maintain the sample chilled. Preweigh aliquots and freeze or extract as soon as possible.

A. **HYPOXANTHINE - ENZYMATIC METHOD (Hx-1)**

APPARATUS (Hx-1)

1. **Food processor** or blender.

2. **Filter paper** Whatman #1.

3. **Spectrophotometer** set at 290 nm.

4. **Water bath** set at 37°C.

5. **pH meter.**

6. **Glassware:** 150 ml beaker, disposable 18 x 150 mm culture tubes, 100 ml and 1000 ml volumetric flasks, 10 mm silica cuvettes (for UV range).

REAGENTS (Hx-1)

1. **Perchloric acid** (6%): Into a 150 ml beaker, carefully weigh 85.7 g of 70% reagent grade $HClO_4$. With caution, slowly pour the weighed perchloric acid into a 1000 ml volumetric flask containing approximately 700 ml distilled water. Make up to volume.

2. **Xanthine oxidase:** Dilute a portion of 10 mg/ml commercial xanthine oxidase (Boeringer Mannheim Ltd., XOD, approximately 0.4 U/mg) in a ratio of 1:50 with 0.05 M phosphate buffer. Add buffer very gradually to avoid loss of enzyme activity. Dilution should be done immediately before use; diluted enzymes may be kept frozen for up to 6 months.

3. **Phosphate buffer** (0.05 M, pH 7.6): Dissolve 17.01 g potassium dihydrogen orthophosphate in about 250 ml distilled water. Adjust to pH 7.6 with 1 M NaOH and dilute to 500 ml with distilled water. A further five-fold dilution with distilled water will give a final concentration of 0.05 M.

4. **Potassium hydroxide/phosphate buffer** (pH 7.6): Dissolve 27.22 g potassium dihydrogen orthophosphate in about 250 ml water

and add 171 ml of 1 M sodium hydroxide. Adjust pH with orthophosphoric acid or sodium hydroxide if necessary. Then add 557 ml of 1 M KOH and make up to 1 litre with water.

5. **Hypoxanthine standards:** In a 100 ml volumetric flask, dissolve 5.0 mg (.005 g) hypoxanthine in 100 ml distilled water. Overnight stirring or agitation in an ultrasonic bath may be necessary for complete dissolution.

PROCEDURE (Hx-1)

a. Preparation of standard curve

1. Prepare the following set of test tubes:

Tube No.	Hx. Std. (ml)	H_2O (ml)	Buffer (ml)	Enzyme (ml)	Conc. Hx. (µg)
1	0.2	2.3	2.0	0.5	10
2	0.4	2.1	2.0	0.5	20
3	0.6	1.9	2.0	0.5	30
4	0.8	1.7	2.0	0.5	40
5	1.0	1.5	2.0	0.5	50
6	0.2	2.8	2.0	0	-
7	0	2.5	2.0	0.5	-
8	0	3.0	2.0	0	-

2. Incubate the standards in a water bath at 37°C for 30 minutes.

3. Measure the absorbance of all standards in 10 mm cuvettes at 290 nm.

4. Calculate the increase in absorbance, Abs, for standards in tubes 1, 2, 3, 4 and 5, as follows:

For tube 1, $Abs = A_1 + A_6 - A_7 - A_8$

Where A_1 = absorbance of tube 1,
A_6 = absorbance of tube 6, etc.

Similarly, calculate Abs for tubes 2, 3, 4 and 5, substituting A_2, A_3, A_4, or A_5 for A_1.

5. Plot Abs for tubes 1 to 5 against hypoxanthine concentration (µg) for tubes 1 to 5. Typical standard curve is shown in Figure 11.1 below:

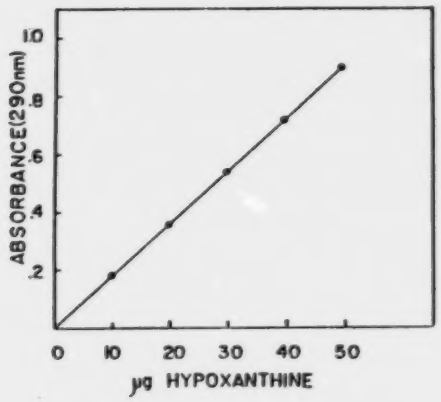


Figure 11.1 Typical standard curve for hypoxanthine

6. Linearity of the standards should be good (r^2 = 0.98 - 0.99). If not, prepare fresh xanthine oxidase solution.

b. Preparation of perchloric acid extracts

1. Blend a preweighed portion (50 g) of homogenized fish for 2 minutes with four times the volume of 6% $HClO_4$ (200 ml).

2. Let the mixture settle for a few minutes.

Caution: Perchloric acid is explosive and can ignite when spilled on cellulose materials (eg. wood, paper, etc.)

3. Filter the extracts through a fluted Whatman #1 filter paper. Collect 50 ml aliquots which may be frozen at -30°C after neutralization for later analysis.

c. Neutralization of extracts

1. Prior to analysis, neutralize 10 ml aliquots of the perchloric acid filtrates to between pH 7.0 and 7.6 by carefully adding an equal volume (10 ml) of potassium hydroxide-phosphate buffer solution. Caution should be exercised in adjusting the pH of extracts since loss of amines can occur at basic pH.

2. Filter the neutralized extracts through fluted Whatman #1 paper.

3. The neutralized extracts are not stable for long periods of time under refrigeration, so prompt analysis is essential. However, neutralized extracts may be frozen at -30°C.

d. Determination of hypoxanthine content in samples

1. Prepare the following set of test tubes for each sample extract:

   Tube A: 1 ml neutralized extract + 2 ml buffer + 2 ml water

   Tube B: 1 ml neutralized extract + 2 ml buffer + 1.5 ml water + 0.5 ml enzyme

   If very high or very low hypoxanthine concentrations are expected, alter the volume of extract and the volume of water, so that the total remains at 5 ml.

2. Incubate the samples in a water bath at 37°C for 30 minutes.

3. Measure absorbance of samples at 290 nm in 10 mm silica cuvettes.

4. Calculate the increase in absorbance, Abs, for each sample as follows:

   $Abs = B + A_8 - A_7 - A$

   A = absorbance from sample tube A
   A_8 = absorbance from tube 8 used in standard curve preparation
   A_7 = absorbance from tube 7 used in standard curve preparation
   B = absorbance from sample tube B

CALCULATIONS (Hx-1)

Hypoxanthine content, expressed as moles/g fish, is:

$$Hx = \frac{H \times [V_1+(0.01 \times M \times W)]}{V_4 \times W} \times \frac{V_2+V_3}{V_3} \times \frac{1}{G} \quad (1)$$

Where,

H = µg Hx from standard curve (Figure 11.1 for example)
M = moisture content of fish expressed in percent
V_1 = volume (ml) of perchloric acid used in 1:4 extraction
V_2 = volume (ml) of KOH/phosphate buffer used for neutralization
V_3 = volume (ml) of extract neutralized by KOH/phosphate buffer
V_4 = volume (ml) of sample extract added to test tube
W = weight (g) of sample used in 1:4 extraction
G = gram molecular weight Hx, i.e. 136.1

EXAMPLE (Hx-1)

A 50 gram (W) sample of whiting flesh was blended with 200 ml (V_1) perchloric acid and analyzed for hypoxanthine content. A 10 ml aliquot of the perchloric acid extract (V_3) was neutralized with 10 ml of KOH-buffer (V_2) solution and 1 ml of the neutralized extract (V_4) was added to a test tube, analyzed and gave a hypoxanthine content of 35.7 ug (H) from the standard curve. The moisture content of the fish was 80.5% (M). Hypoxanthine content may be calculated from formula (1) as:

$$Hx = \frac{35.7 \times [200+(0.01 \times 80.5 \times 50)]}{1 \times 50} \times \frac{10+10}{10} \times \frac{1}{136.1}$$

$$= 171.54 \times \frac{20}{10} \times \frac{1}{136.1}$$

$$= 2.52 \text{ µ moles/g fish}$$

B. HYPOXANTHINE - L.C. METHOD (Hx-2)

APPARATUS (Hx-2)

1. **Food processor.**
2. **Filter paper** Whatman #1.
3. **Blender.**
4. **LC pump** - operated at 1.0 ml/min.

5. **LC injector** - loop injector fitted with 20 µl loop.

6. **Detector** - UV detector operated at 254 nm.

7. **Recorder** - strip chart

8. **LC Column** - RP-8 MPLC reverse phase analytical column 4.6 mm id x 10 cm, 10 µM particle size fitted with an RP-8 MPLC guard column 4.6 mm id x 3 cm, 10 M particle size (Brownlee Labs. Inc. Santa Clara, CA).

9. **Aqueous sample clarification kit** - PN 26865 used to remove fine particles of 0.45 µM or greater (Water Associates, Inc., Milford, MA).

10. **Glassware** - 10, 50, 500 and 1000 ml volumetric flasks, 200 ml blender flasks, screw topped test tubes (16 x 125 mm).

REAGENTS (Hx-2)

1. **Perchloric acid (0.6 M):** with caution slowly add 32.3 ml concentrated perchloric acid (60%) to a 500 ml volumetric flask containing approximately 400 ml distilled water. Make up to volume.

2. **Potassium hydroxide/phosphate buffer (pH 7.6):** dissolve 8.16 g KH_2PO_4 in approximately 60 ml distilled water and adjust to pH 7.6 with 50% KOH. Dilute to 100 ml with distilled water.

3. **50% KOH:** dissolve 50 g KOH in 50 ml distilled water and cool to room temperature.

4. **LC mobile phase:** 0.01 M potassium phosphate buffer pH 4.5. Dissolve 1.36 g KH_2PO_4 in approximately 400 ml double-distilled water. Adjust to pH 4.5 with KOH or H_3PO_4 as necessary and dilute to 1 litre.

5. **Standards:** (a) Stock solutions - prepare individual standards for LC by dissolving 0.010 g Hx, INO, AMP and IMP respectively in 40 ml distilled water. Vigorous stirring or overnight agitation may be necessary for complete dissolution of Hx. Dilute to the mark in 50 ml volumetric flasks. (Standards are diluted 1:10 before injection on the LC).
(b) Mixed standards (working solutions) - pipet 0.125, 0.250, 0.375 and 0.50 ml of each stock solution of IMP and Hx into four separate 10 ml volumetric flasks. Add 0.250, 0.50, 0.75 and 1.0 ml respectively of each stock solution of AMP and INO to the flasks. Dilute to the mark with distilled water. These four solutions contain 2.5, 5.0, 7.5 and 10 µg/ml solution of IMP and Hx and 5.0, 10, 15 and 20 µg/ml solutions of AMP and INO. Mixed standards solutions are stable for approximately 2 weeks when stored at 0-4°C.

PROCEDURE (Hx-2)

a. Preparation of standard curve

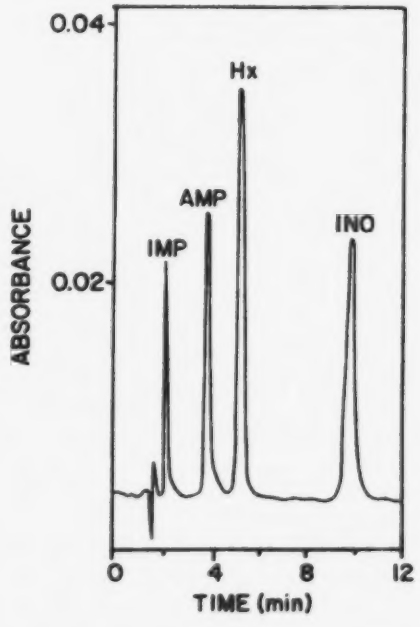


Figure 11.2 LC UV (254 nm) trace of separation of standard IMP, Hx (50 mg each), AMP and INO (100 mg each) run on a Brownlee MPLC RP-8 (10 um, 4.6 mm id x 10 cm, developed with 0.01M KH_2PO_4 buffer, pH 4.5 at 1.0 mL/min) reverse phase analytical column.

1. Set flow rate of mobile phase at 1.0 ml/min and let column and detector equilibriate 20-25 minutes.

2. Inject 10 µl aliquots of each mixed standard solution.

3. Determine absorbance of the various nucleotides from peak heights recorded at 254 nm (Figure 11.2).

4. Plot peak height vs µg injected to provide a standard curve.

5. Prepare standard curves at least twice per day to assure accurate quantitation.

b. **Sample Preparation**

1. Without thawing, blend a preweighed 5 g portion of homogenized fish at maximum speed for 2 minutes with ten times the volume of 0.6 M $HClO_4$ (50 ml).

2. Suction-filter contents through Whatman #1 paper using a small amount of distilled water to rinse the blender flask.

3. Mix the filtrate well and note the volume (V_1) with graduated cylinder.

4. Transfer a 1.0 ml aliquot of the filtrate to a screw-top test tube containing 1.0 ml KOH phosphate buffer (pH 7.6). Mix the solution, cool to 0-4°C then decant and filter through the aqueous clarification kit.

5. Inject aliquots (V_2) of neutralized filtrate directly for LC analysis.

6. Dilute with distilled water if necessary (D).

CALCULATION

Hx content in fish tissue is calculated from the following equation:

$$\text{Hx content } (\mu \text{ moles/g}) = KPV_1 D/HV_2W \quad (1)$$

where:

P = peak height (mm)
H = slope of standard curve (mm/ug)
V_1 = total volume of perchloric extract plus wash (ml)
D = dilution factor of neutralized extract before LC
V_2 = injection volume for LC (µl)
W = weight of sample (g)
K = 14.71 (µl) (µ moles)/(µg) (ml) a constant which takes into account the 1:1 dilution during neutralization.

IMP, AMP and INO content in fish tissues may be calculated using equation 1 but with the following K values: 5.75, 5.76 and 7.46 (µl) (µ moles)/(µg) (ml) respectively.

EXAMPLE (Hx-2)

A 4.93 gram (W) sample of cod flesh was blended with 50 ml perchloric acid. The total volume of the perchloric acid extract plus wash was 60 ml (V_1). The slope of the standard curve was 1535 mm/ µ g (H). A 10 ml aliquot (V_2) of the neutralized, clarified filtrate was injected directly onto the LC. No dilution was required (D=1) with a peak height of 150 mm resulting (P). Hx content may be calculated from equation 1 as:

$$Hx = \frac{14.71 \times 150 \times 60 \times 1}{1535 \times 10 \times 493} = \frac{1.32 \times 10^5}{7.57 \times 10^4}$$

$$= 1.74 \text{ µ moles/g fish}$$

REFERENCES

1. ESKIN, N.A., H.M. Henderson, and R.J. Townsen, 1971. Biochemistry of Foods. Academic Press, New York.

2. MARTIN, R.E., R.J.H. Gray, M.D. Pierson, 1978. Quality assessment of fresh fish and the role of naturally occurring microflora. Food Technol. 30:188.

3. JONES, N.R. and J. Murray, 1962. Degradation of adenine and hypoxanthine - nucleotide in the muscle of chill-stored trawled cod (Gadus callarias). J. Sci. Food Agric. 13:475.

4. JONES, N.R., J. Murray, E.I. Livingstone and C.K. Murray, 1964. Rapid estimations of hypoxanthine concentrations as indices of the freshness of chilled-stored fish. J. Sci. Food Agric. 15:763.

5. FRASER, D.I., S.G. Simpson, and W.J. Dyer, 1968. Very rapid accumulation of hypoxanthine in the muscle of redfish stored in ice. J. Fish. Res. Board Can. 25:817.

6. KASSEMSARN, B., B. Sanz Perez, J. Murray and N.R. Jones, 1963. Nucleotide degradation in the muscle of iced haddock (Gadus aeglefinus), lemon sole (Pleuronectes microcephalus) and plaice (Pleuronectes platessu). J. Food Sci. 28:28.

7. EHIRA, S. and H. Uchiyama, 1969. Rapid estimation of freshness of fish by nucleotide phosphorylase and xanthine oxidase. Bull. Jap. Soc. Sci. Fish. 35:1080.

8. HUGHES, R.B. and N.R. Jones, 1966. Measurement of hypoxanthine concentration in canned herring as an index of the freshness of the raw material with a comment on flavour relations. J. Sci. Fd. Agric. 17:434.

9. SPINELLI, J., G. Pelroy, and D. Miyauchi, 1969. Quality indices that can be used to assess irradiated seafoods. In "Freezing and Irradation of Fish", ed. R. Kreuzer. Fishing News (Books) Ltd., London, U.K., p. 425.

10. BLIGH, E.G., (1971). Specific problems in the quality assessment of freshwater fish. In "Fish Inspection and Quality Control", ed. R. Kreuzer. Fishing News (Books) Ltd., London, U.K., p. 81.

11. SPINELLI, J., M. Eklund and D. Miyauchi, 1964. Measurement of hypoxanthine in fish as a method of assessing freshness. J. Food Sci. 79:710.

12. BEUCHAT, L.R., 1973. Hypoxanthine measurement in assessing freshness of chilled channel catfish (Ictalurus punctatus). J. Agric. Food Chem. 21:453.

13. JAHNS, F.D., J.L. Howe, R.J. Coduri and A.G. Rand, 1976. A rapid visual enzyme test to assess fish freshness. Food Technol. 30:27.

14. BURT, J.R., 1977. Hypoxanthine: A biochemical index of fish quality. Process. Biochem. 12:32.

15. BOYD, N.G. and N.D.C. Wilson, 1977. Hypoxanthine concentrations as an indicator of freshness of iced snapper. N.Z.J. Food. Sci. 20:139.

16. JAHNS, F.D. and A.G. Rand, Jr., 1977. Enzyme methods to assess marine food quality. In Enzymes in Food and Beverage Processing. R.L. Ory and A.J. St. Angelo (eds). ACS Symp. Ser. No. 47. AVI Publishing, Westport, CT.

17. SAITO, T., A. Arai and M. Matsuyoshi, 1959. A new method for estimating the freshness of fish. Bull. Jap. Soc. Sci. Fish. 24:749.

18. WATANABE, E., K. Ando, I. Karube, H. Matsuoka, and S. Suzuki, 1983. Determination of hypoxanthine in fish meat with an enzyme sensor. J. Food Sci. 48:496.

19. BURT, J.R., D.M. Gibson, A.G. Jason and H.R. Sanders, 1976. Comparison of methods of freshness assessment of wet fish. II. Instrumental and chemical assessments of boxed experimental fish. J. Food Technol. 11:73.

20. BURT, J.R., G.D. Stroud and N.R. Jones, 1969. Estimation of hypoxanthine concentrations in fish muscle by a rapid, visual modification of the enzymatic assay. In Fishing and Irradiation of Fish. R. Kreuzer (Ed.). Fishing News (Books) Ltd., London.

21. JACOBER, L.F. and A.G. Rand, Jr., 1982. Biochemical evaluation of seafood. In

Chemistry and Biochemistry of Marine Food Products (eds. R.E. Martin, G.J. Flick, C.E. Hebard and D.R. Ward) AVI Pub., Conn. pp. 347-365.

22. SPINELLI, J., 1967. Degradation of nucleotides in ice-stored halibut. J. Food Sci. 32:38.

23. Analytical Methods Committee, 1979. Recommended General Methods for the Examination of Fish and Fish Products. Analyst 103:434.

24. BURT, J.R., J. Murray and G.D. Stroud, 1968. An improved automated analysis of hypoxanthine. J. Food Technol. 3:165.

25. JAHNS, F.D. and A.G. Rand, 1978. Immobilized enzyme method to assess fish quality. U.S. Patent 4, 105, 800.

26. UPDIKE, S.J. and G.P. Hicks, 1967. The enzyme electrode. Nature 214:986.

27. JONES, N.R., 1960. The separation and determination of free purines, pyrimidines and nucleosides in cod muscle. Analyst 85:111.

28. JONES, N.R. and J. Murray, 1964. Rapid measures of nucleotide dephosphorylation in iced fish muscle. Their value as indicies of freshness and of inosine 5'-monophosphate concentration. J. Sci. Food. Agri. 15:684.

29. FRASER, D.J., J.R. Dingle, J.A. Hines, S.C. Nowlan and W.J. Dyer, 1967. Nucleotide degradation, monitored by thin-layer chromotography and associated postmortem changes in relaxed cod muscle. J. Fish. Res. Board Can. 24:1837.

30. BURNS, B.G. and P.J. Ke, 1985. Liquid chromotrographic determination of hypoxanthine content in fish tissue. J. Assoc. Off. Anal. Chem. 68:144.

31. FRASER, D.I., D.P. Pitts, and W.J. Dyer, 1968. Nucleotide degradation and organoleptic quality in fresh and thawed mackerel muscle held at and above ice temperature. 25:239.

12. SIMULTANEOUS DIMETHYLAMINE (DMA)/ TRIMETHYLAMINE (TMA) DETERMINATION

GENERAL DISCUSSION (see methods 9 and 18 for single TMA/DMA determinations)

Many marine species contain trimethylamine oxide (TMAO) a compound which functions physiologically in a role similar to that of urea or uric acid in land animals, i.e. TMAO is excreted to maintain nitrogen balance (1). The compound is rarely found or absent in freshwater fish. It is highest in elasmobranchs sharks, of intermediate levels in teleosts (bony fish) and very low in bivalves (2, 3). Among the teleosts, the gadoid family (cod, pollock, haddock, whiting, hake and cusk) contains the highest levels of TMAO while flatfish (plaice, flounder, sole, etc) have the least (1). Freshwater fish contain negligible levels of TMAO (4).

By-products of TMAO degradation are organoleptically evident during both iced or chilled and frozen storage. Where bacterial activity is held in check (frozen storage), TMAO is broken down by a naturally occuring enzyme TMAO-ase to equimolar amounts of dimethylamine (DMA) and formaldehyde (FA) (1, 4). As with TMAO levels, various species exhibit different amounts of TMAO-ase activity, the gadoids being among the highest. Enzyme activity is maximum at temperatures near -10°C and becomes slower with decreasing temperatures (5, 6). The presence of formaldehyde causes decreased protein extractability, inferior texture and generally poor organoleptic qualities (7, 8, 9, 10, 11, 12, 1, 4). Since formaldehyde is difficult to extract quantitatively (13), the DMA value is often recognized as an indicator of frozen quality in species where high TMAO-ase activity is present (14).

Under refrigerated conditions (and warmer), bacterial enzymes, generally from Alteromonas putrefaciens, degrade TMAO to produce the ammonia-like odoriforous compound trimethylamine (TMA). The level of TMA has been widely accepted as an indicator of bacterial and eating quality in marine fish (15, 16, 4, 17).

The original published picrate methods for TMA analysis by Dyer (18, 19, 20) were found to be subject to DMA interference (21). The importance had been originally discounted since the TMA method was for fish in chilled and not frozen storage (18). In reality however, frozen fish may contain both TMA and DMA. If DMA were at extremely high levels, of 10 mg percent for example, a TMA quantity of 2.1 mg percent would be indicated using the original K_2CO_3 method (21). Substitution of 25% KOH for K_2CO_3 eliminated most of the DMA interference (21). Later it was discovered that TMA caused erroneous values in the dithiocarbamate DMA analysis (22) of Dyer (18).

The difference in molar extinction coefficients observed for TMA and DMA with K_2CO_3 and KOH in the picrate method was a basis for the simultaneous DMA/TMA analysis proposed by Castell and co-workers (14). Since various experimental parameters (shaking, especially) were found to affect the results (14, 22) individual laboratories should establish "in-house" standard curves.

a. **Application**

The simultaneous TMA/DMA test is applicable to the determination of DMA and TMA in fish stored for long periods in a chilled condition followed by frozen storage, especially long term, at temperatures promoting accelerated DMA formation (generally above -30°C). Traditional TMA or DMA analyses of such material would be subject to interference. Species particularly vulnerable to DMA production are hake, cusk, pollock, and to a lesser degree, cod. Where only one amine (TMA or DMA) is present, the described single laboratory methods 9 or 18 should be used rather than the simultaneous method.

b. **Principle**

Amines, primary, secondary, and teriary, react with picric acid to produce yellow colored picrates which are extractable in toluene. With K_2CO_3, five times as much DMA as TMA is required to produce a color

reaction (absorbance 410 nm) with picric acid (9); with KOH a much greater amount is required (14). The varied degree of reactivity of DMA and TMA in KOH or K_2CO_3 is quantified in a set of standard curves. The proportions of TMA and DMA are the determined in an unknown sample by analyzing with both KOH and K_2CO_3 and solving a simultaneous equation. Addition of formaldehyde minimizes interference from ammonia.

c. **Precautions**

1. Shaking reproducibility is critical. Deviation in procedure will alter the standard curve.

2. Standard curves (first and second analysis) may be prepared and stored on file as "in-house" data. They should be rechecked whenever changes are incorporated (new equipment or personnel).

3. The same volume of extract must be used for "first" and "second" analysis.

4. Temperature during mixing and shaking of solutions of TCA extract, formaldehyde, alkali, and toluene should be maintained at 30°C to ensure reproducibility (21). A water bath at this temperature should be at hand.

5. Only a small amount of picric acid should be dried for use. Picric acid will detonate if subjected to shock when in a dry state. Stock bottle should be kept moist with distilled water.

6. Toluene used in the analysis should be dried over $NaSO_4$ or molecular seives.

7. Picric acid reagent should be added to clean dry test tubes since the method is sensitive to water and other contaminants.

SAMPLE PREPARATION

A representative sample should be comminuted in a cuisinart food processor and 50-100g aliquots preweighed. These should be used immediately, maintained on ice (short time) or frozen at -35°C (few days only). Care must be taken to prevent further TMA or DMA formation before analysis.

APPARATUS

1. **Blender,** with small blender jar

2. **Low speed centrifuge** (optional)

3. **Rotator,** eg. Cole-Parmer Model 7621-20

4. **Spectrophotometer** for use at 410 nm

5. **Filter paper** Whatman #4 or glass wool

6. **Glassware:** Funnels, 50 ml Erlenmeyer flasks, screw capped (Teflon lined) culture tubes (20 x 150 mm)

REAGENTS

1. **Stock TMA Standard (1.00 mg TMA-N/ml):** Dissolve 0.6820 g TMA-HCl weighed to nearest 0.0001 g (dried overnight in desiccator) in distilled water. Dilute to 100 ml. Refrigerate.

2. **Working TMA Standard (0.01 mg TMA-N/ml):** Pipet 1 ml **stock** TMA solution to a 100 ml volumetric flask and dilute to volume with distilled water.

3. **Stock DMA standard (1.0 mg DMA-N/ml):** In a 100 ml volumetric flask, dissolve 0.5830 g dried DMA-HCl weighed to nearest 0.0001 g in a small amount of distilled water. Make up to 100 ml with distilled water. Refrigerate.

4. **Working DMA standard (0.01 mg TMA-N/ml):** Pipet 1 ml **stock** DMA standard to a 100 ml volumetric flask and dilute to volume with distilled water. Refrigerate.

5. **Formaldehyde (10%):** Dilute 26.8 ml formalin (37.3% HCHO) to 100 ml with distilled water.

6. **Potassium hydroxide (25% w/w):** Dissolve 25 g KOH in 75 ml distilled water. Cool.

7. **Potassium carbonate (50% w/w):** Dissolve 50 g K_2CO_3 in 50 ml distilled water.

8. **Trichloroacetic acid (7.5% w/w):** Dissolve 7.5 g TCA in 92.5 ml distilled water.

9. **Moisture-free toluene:** Dry over anhydrous Na_2SO_4. Not necessary to distill.

10. **Anhydrous sodium sulfate, granular**

11. **Stock picric acid reagent:** Dissolve 2.0 g picric acid (dried overnight at room temperature in desiccator) in moisture-free toluene. Dilute to 100 ml with dried toluene.

    N.B. Picric acid should be kept moist at all times to prevent explosion. When preparing standard only a small amount should be dried and caution should be exercised in handling.

12. **Working picric acid reagent:** Pipet 1 ml **stock** picric acid reagent to a 100 ml volumetric flask and dilute to volume with dried toluene.

PROCEDURE

A. Preparation of Extracts

1. Add 50 g sample and 100 ml of 7.5% TCA (1:2 extraction) to a small blender jar and blend thoroughly.

2. Centrifuge at 4°C for 15 minutes at about 2000 x g (approximately 4000 rpm). An alternate purification procedure to centrifugation and decanting is filtration through Whatman #4 filter paper.

3. Decant supernatant fluid through glass wool.

4. Extracts may be frozen at -20°C for extended periods of time with little deterioration.

B. Analysis of TCA extracts

Method Note: Samples and standards must be analyzed for amines by **both** KOH and K_2CO_3 procedures which follow:

a. First analysis - KOH method

1. Pipet aliquots (0.1-4 ml) of extract into a 20 x 150 mm screw capped tube. Concentrations of TMA in extract will determine appropriate aliquot size.

2. Prepare separate **standards for both TMA and DMA** by pipetting 0, 0.5, 1.0, 1.5, 2.0 and 2.5 ml of each working standard (DMA or TMA) solution into individual 20 x 150 mm screw capped tubes to represent 0, 0.005, 0.01, 0.015, 0.020 and 0.025 mg amine nitrogen.

3. Add water to total volume of 4.0 ml for both extracts and standards. For a blank use 4.0 ml distilled water.

4. Then add 1 ml 10% formaldehyde, 10 ml toluene, and 3.0 ml 25% KOH to each. Ensure temperature is at 30°C (see precautions note #3).

5. Cap tubes and mix for 15 min on rotator.

6. With pasteur pipette, remove ca 7 ml of upper toluene layer to a large drying tube containing approximately 0.3 to 0.4 g anhydrous Na_2SO_4. Shake gently (vortex mixer) until the solution is clear.

7. Pipet 5 ml of picric acid working reagent into clean dry test tubes. If color change is evident when reagent is added to empty tubes (yellow), contamination is present and clean test tubes should be obtained.

8. Pipet 5 ml of toluene solution (from Step 6) into tubes containing picric acid reagent and mix by swirling gently.

9. Measure absorbance at 410 nm.

b. Second analysis - K_2CO_3 method

Proceed as in first analysis (KOH) above, but substitute 50% K_2CO_3 for 25% KOH in step 4. Continue as for KOH method.

CALCULATIONS

1. Produce 4 standard curves for 0, 0.005, 0.01, 0.015, 0.020, and 0.025 mg DMA-N or TMA-N by the KOH and K_2CO_3 methods, plotting absorbance at 410 nm versus mg amine - nitrogen added (see Figure 12.1 below).

2. Label the curves as follows:
   a = DMA curve determined by K_2CO_3 method (second analysis)
   b = TMA curve determined by K_2CO_3 method (second analysis)
   c = DMA curve determined by KOH method (first analysis)
   d = TMA curve determined by KOH method (first analysis)

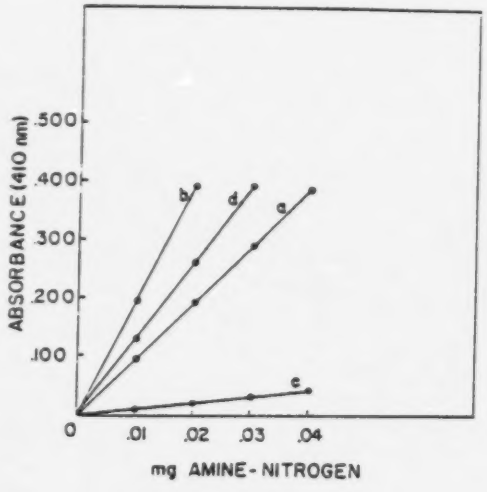


Figure 12.1 Typical standard curves for TMA and DMA by first and second analyses: curve a = DMA curve by second analysis (K_2CO_3); curve b = TMA curve by second analysis (K_2CO_3); c = DMA curve by first analysis (KOH); and d = TMA curve by first analysis (KOH).

3. Define the following values from the standard curves:

   A = absorbance from curve a at 0.01 mg amine-nitrogen

   B = absorbance from curve b for 0.01 mg amine-nitrogen

   C = absorbance from curve c for 0.01 mg amine-nitrogen

   D = absorbance from curve d for 0.01 mg amine-nitrogen

4. Using this information calculate DMA (K_1) and TMA (K_2) content of the sample tubes by the formulae:

$$K_1 = \frac{R - S}{A/B - C/D} \qquad (1)$$

and

$$K_2 = S - \frac{C \times K_1}{D} \qquad (2)$$

Where:
A, B, C, and D are defined in step 3 of calcuations above
K_1 = mg DMA-N in tube
K_2 = mg TMA-N in tube
R = amine value (mg) from curve b corresponding to absorbance of sample obtained in second analysis (K_2CO_3) method)
S = amine value (mg) curve d corresponding to absorbance of sample obtained in first analysis (KOH method)

5. Calculate final concentration of DMA and TMA as mg per 100 g fish by:

$$\text{DMA-N} = \frac{K_1 \times [V_1 + (0.01 \times M \times W)]}{V_2 \times W} \times 100 \qquad (3)$$

Where:
K_1 = mg DMA-N from formula 1
M = moisture content of fish sample expressed in percent
V_1 = volume in ml of TCA added for 1:2 extraction
V_2 = volume in ml of extract added to test tube
W = weight of fish used in 1:2 extraction

Similarily,

$$TMA\text{-}N = \frac{K_2 x\ [V_1 + (0.01 \times M \times W)]}{V_2 \times W} \times 100 \quad (4)$$

Where: symbols are as defined above

EXAMPLE

A commercial sample of cod flesh of 80.8% moisture (M) was analyzed by the simultaneous TMA/DMA procedure. a portion, 50 g (W) was blended with 100 ml TCA (V_1) solution. Aliquots of 0.3 ml (V_2) analyzed by "first" and "second" analyses produced absorbance values of 0.19 and 0.33, respectively.

From standard curves which were experimentally derived (Figure 12.1), the values for (i.e. absorbance for 0.01 mg amine-nitrogen) A, B, C, and D defined by the graphs were:

A = 0.100
B = 0.210
C = 0.010
D = 0.149

From curves d and b, absorbance values of 0.19 and 0.33 corresponded to S and R (amine values) of:

S = 0.014 mg amine
R = 0.016 mg amine

By formula 1,

$$K_1 = \frac{0.016 - 0.014}{\frac{0.100}{0.210} - \frac{0.010}{0.149}}$$

$$= \frac{0.002}{0.476 - 0.067}$$

= 0.0049 mg DMA-N

By formula 2,

$$K_2 = 0.014 - \frac{0.010 \times 0.0049}{0.149}$$

$$= 0.014 - 0.0003$$

= 0.0137 mg TMA-N

Data from the extraction and analysis of the sample were:

M = 80.8
V_1 = 100 ml
V_2 = 0.3 ml
W = 50 g

Thus, DMA-N expressed as mg per 100 g fish (from formula 3) is:

$$DMA = \frac{0.0049 x [100 + (0.01 \times 80.8 \times 50)]}{0.3 \times 50} \times 100$$

$$= \frac{0.0049 \times 140.4}{15} \times 100$$

= 4.59 mg DMA-N/100 g fish

And, similarily, TMA-N expressed as mg per 100 g fish (from formula 4) is:

$$TMA = \frac{0.0137 x [100 + (0.01 \times 80.8 \times 50)]}{3 \times 10} \times 100$$

$$= \frac{0.0137 \times 140.4}{15} \times 100$$

= 12.8 mg TMA-N/100 g fish

REFERENCES

1. SHENOUDA, S.Y.K. 1980. Theories of protein denaturation during frozen storage of fish flesh. In Advances in Food Research, Academic Press, Inc. Vol. 26:275.

2. KONOSU, S., K. Watanabe, and T. Shimizu. 1974. Distribution of nitrogenous constituents in the muscle extracts of eight species of fish. Bull. Jap. Soc. Sci. Fish. 40:909.

3. SUYAMA, M., and H. Suzuki. 1975. Nitrogenous constituents in the muscle extracts of marine elasmobranchs. Bull. Jap. Soc. Sci. Fish. 41:787.

4. HEBARD, C.E., G.J. Flick, and R.E. Martin. 1982. Occurrence and significance of trimethylamine oxide and its derivatives in fish and shellfish. In Chemistry and Biochemistry of Marine Food Products (R.E. Martin, G.J. Flick, C.E. Hebard, and D.R. Ward, eds.) AVI Pub. Conn. pg 149.

5. AMANO, K., and K. Yamada. 1965. The biological formation of formaldehyde in cod flesh. In FAO - The Technology of Fish Utilization (R. Kreuzer, Ed.) Fishing News (Books), London. pg. 73.

6. AMANO, K., and K. Yamada. Biological formation of formaldehyde in the muscle tissue of gadoid fish. Bull. Jap. Soc. Sci. Fish. 30:430.

7. GILL, T.A., R. A. Keith, and B. Smith-Lall. 1979. Textural deterioration of red hake and haddock muscle in frozen storage as related to chemical parameters and changes in the myofibrillar proteins. J. Food Sci. 44:661.

8. DINGLE, J.R. and J.A. Hines, 1975. Protein instability in minced flesh from fillets and frames of several Atlantic fishes during storage at -5°C. J. Fish Res. Bd. Canada 32:775.

9. CASTELL, C.H., W. Neal, and B. Smith. 1970. Formation of dimethylamine in stored frozen sea fish. J. Fish. Res. Bd. Canada 27:1685.

10. HILTZ, D.F., W.J. Dyer, and D.W. Lemon. 1974. Some properties of fillets and minced flesh of silver hake (Merluccius hibinearis) in frozen storage. Environment Canada, Fisheries and Marine Service, New Series Circular No. 46.

11. DYER, W.J., and D.F. Hiltz. 1974. Sensitivity of hake muscle to frozen storage. Environment Canada, Fisheries and Marine Service, New Series Circular No. 45.

12. SIKORSKI, Z., J. Olley, and S. Kostuch. 1967. Protein changes in frozen fish. In Critical Reviews in Food Science and Nutrition 8:97.

13. OTA, F. 1958. Carbonyl compounds in fish as related to the deterioration. I. Detection of volatile carbonyl compounds formed in fish flesh. Jap. Soc. Sci. Fish. 24:334.

14. CASTELL, C.H., B. Smith, and W.J. Dyer. 1974. Simultaneous measurements of trimethylamine and dimethylamine in fish, and their use for estimating quality of frozen stored gadoid fillets. J. Fish. Res. Bd. Canada 31:383.

15. CASTELL, C.H. 1970. Current status of the TMA test as a measure of spoilage in fish. Fish. Res. Bd. of Canada, Halifax, N.S. New Series Circular No. 38.

16. BULLARD, F.A., and J. Collins. 1980. An improved method to analyze trimethylamine in fish and the interference of ammonia and dimethylamine. Fishery Bulletin 78:465.

17. SHEWAN, J.M., and N.R. Jones. 1957. Chemical changes occurring in cod muscle during chill storage and their possible use as objective indices of quality. J. Sci. Food. Agric. 8:491.

18. DYER, W.J., and Y. A. Mounsey. 1945. Amines in fish muscle II. Development of trimethylamine and other amines. J. Fish. Res. Bd. Canada 6:359.

19. LOVE, R.M. 1970. The Chemical biology of fishes. Academic Press, New York.

20. DYER, W.J. 1959. Report on Trimethylamine in fish. J. AOAC 42:292.

21. TOZAWA, H., K. Enokihara, and K. Amano. 1971. Proposed modification of Dyer's methods for trimethylamine determination in cod fish. In Fish Inspection and Quality Control. R. Kreuzer (ed) Fishing News (Books) Ltd. London p 187-190.

22. MORAL, A., F. Jimenez-Colmenero, and A.J. Borderias. 1979. Contribution to the dimethylamine and trimethylamine measurement in frozen fish using Dyer's method. Proc. XV Int. Congress of Refrig. Vo. III:1053.

13. 2-THIOBARBITURIC ACID TEST (TBA/TBARS)

GENERAL DISCUSSION

Fatty fish under conditions of frozen storage lose quality through oxidative deterioration of their lipid components. Susceptibility and rate of oxidation depends to a large extent on fatty acid profile; the greater the unsaturation, the more easily will a fat oxidize (11). Other factors, many still unknown, contribute to oxidation. The skin and dark meat of mackerel (Scomber scombrus) for example, are particularly susceptible to oxidation (2) while herring (Clupea harengus) of similar unsaturation appear more resistant. Accumulation of oxidation by-products is responsible for objectionable flavours perceived in "rancid" foods.

Oxidation is a free-radical process proceeding through initiation, propagation, and termination steps (3) and yields aldehydes, acids, epoxides, diglycerides, monoglycerides and polymers (4). However, since end products are continually being lost to further reaction, measurement of oxidation degree is difficult. The three most popular laboratory methods applied to the measurement of oxidation in fishery products, namely the 2-thiobarbituric acid test (TBA), peroxide value (POV) and carbonyl value (COV), all suffer from this transitory nature of products, i.e. they increase to a maximum and then decline (5, 6, 7, 8). Therefore, it is usually recommended that at least two tests be used for oxidative quality evaluation, particularly if the free fatty acid content is high. Ultimately, a cooked organoleptic evaluation should be conducted to confirm product quality.

Development of the TBA test has had much scientific input. In 1944 Kohn and Liversidge reported that "animal tissues heated with thiobarbituric acid yield an orange-red product". The test subsequently evolved to include TBA addition directly to tissue (9, 10, 11) to a distillate (12, 13, 14, 15, 16, 17, 18), and to a TCA extract (19, 20). While the TBA test remains popular (21, 22, 23, 24, 25, 26, 27), it is much criticised for its non-specificity (28), the transitory nature of the malonaldehyde being measured (29, 23, 13, 19, 30, 6) and the unreliability of malonaldehyde content as an oxidative indicator in general since the amount of the compound formed depends on the types of unsaturated fatty acids present in the tissue; malonaldehyde is formed only from peroxides derived from fatty acids containing three or more double bonds (31). Numerous compounds have also been found which interfere with the TBA test either by producing other absorbing TBA complexes or by reacting with the malonaldehyde itself (32, 14, 33, 30, 34, 35, 36).

The currently favoured TBA procedure is that of Tarladgis (13) involving adjustment of sample to pH 1.5, distillation and reaction with 2-thiobarbituric acid. The method was subsequently improved to include incorporation of antioxidants and chelating agents (16, 35) to reduce oxidation during distillation. To avoid the time consuming acidification step the procedure was modified for mackerel (Scomber scombrus) to include addition of a large fixed aliquot of acid prior to distillation (17, 18). Values obtained by this method (designated TBARS for TBA reactive substances) are very much lower than those of the original Tarladgis procedure. The latter test although in linear agreement with that of Tarladgis (13) has not been extensively tested or correlated for species other than mackerel. Figure 13.1 illustrates the approximate relationship of TBARS and Tarladgis TBA values (37). Approximate recoveries of malonaldehyde for TBARS and Tarladgis procedures are ca 30 and 60%, respectively. Elasmobrachs (dogfish, sharks, etc.) may exhibit depressed TBA values due to low recoveries of malonaldehyde (19).

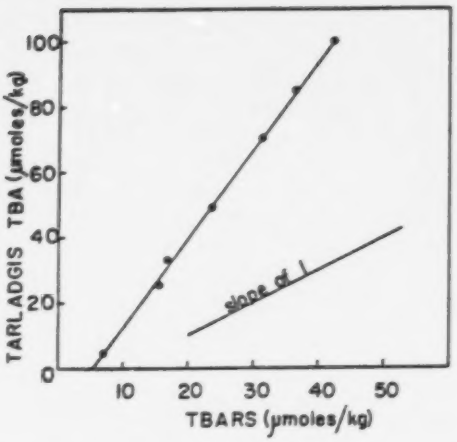


Figure 13.1 Experimentally derived TBA (13) and TBARS (17, 18) relationship for Atlantic mackerel (Scomber scombrus).

a. **Application**

The TBA test is applicable to most fatty fish for the evaluation oxidative quality after frozen storage but should be used with caution with species or product forms for which TBA data is not documented in the literature. Certain species may possess TBA reactive substances which absorb in the 535-538 nm range and are unrelated to oxidative changes. Spectra should be checked on a scanning visible spectrophotometer to ensure no interferring peaks are present for the TBA procedure chosen. For mackerel and herring the TBARS procedure (Method A) may be applied. For other species and product forms (especially fish meal) unless TBARS and Tarladgis correlating data exists, the Tarladgis procedure (Method B) should be used. Since pH 1.5 is sometimes difficult to measure accurately with certain electrodes and the time necessary for pH adjustment causes further oxygen incorporation and oxidation, the more rapid TBARS procedure is often more convenient.

When interpreting data, particularly from long-term stored samples, it should be kept in mind that TBA values attain a maximum value and decline on further storage, that value being dependent on species, accessibility to oxygen (packaging), and storage temperature.

b. **Principle**

The TBA procedure entails distillation of TBA reactive substances (malonaldehyde, other aldehydes, and volatiles) from acidic media (TBARS, pH < 0.5; Tarladgis TBA, pH = 1.5) in the presence of chelating agents and anti-oxidants disodium EDTA and propyl gallate. Subesquent reaction with 2-thiobarbituric acid under acidic conditions to produce red TBA complexes absorbing at 535-538 nm. The condensation reaction with malonaldehyde is illustrated as follows (38):

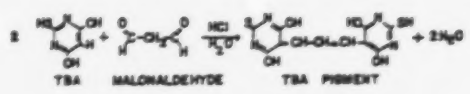


Figure 13.2 Reaction of malonaldehyde with 2-thiobarbituric acid (38).

c. **Precautions**

1. Samples must be protected from oxidation during storage prior to analysis by exclusion of oxygen and low temperature storage (-30°C). The refrigerator freezer compartment should not be used for storage of samples. Do not allow samples to warm during comminution.

2. Consideration must be given to product end use; only if samples would normally be allowed to thaw before cooking should they be analyzed from a thawed state. Thawing will result in slight elevation of measured TBA value.

3. Heating rate should be maximum and performed reproducibly.

4. Distillates may generally be stored overnight without undue change to TBA value.

5. Absorbance should be measured within two hours of color development.

6. The standard TEP (1×10^{-4} M) is stable at refrigerator temperatures for several months.

SAMPLE PREPARATION

Products may be sampled with either minimum rapid defrost or overnight refrigerated thawing. The latter method will elevate TBA values slightly but eliminates the possibility of samples becoming too warm through rapid thawing.

1. Cover or wrap frozen product in plastic to protect from dehydration during defrost and place in refrigerator until just thawed (overnight). For rapid defrost sample may be enclosed securely in a plastic bag and placed in a sink of cold running water until pliable.

2. Excise fillets and rinse briefly.

3. For mackerel, remove the skin membrane in one motion by grasping the membrane at the tail end (of the fillet) and pulling toward the head. Herring fillets are analysed without skin removal.

4. Comminute a representative sampling of fillets (3 or 4) in a Cuisinart food processor or similar comminuting device. If possible flush material with nitrogen while blending.

5. Weigh at least two 10 gram portions from each blend into small disposable sample cups. Flush each with nitrogen and store on ice (briefly) or at -30°C.

APPARATUS

1. **Blender** with small blender jar

2. **Vertical distillation assembly** (see Figures 13.3 or 13.4)

3. **Water bath,** boiling (100°C)

4. **Spectrophotometer** visible range, 538 nm

5. **pH meter,** with combination pH electrode (for Method B)

6. **Glassware:** 50 ml volumetric flask, 15x125 mm screw capped (Teflon lined) test tubes, 500 ml round bottom flasks to fit distillation assembly, 250 ml beakers (Method B)

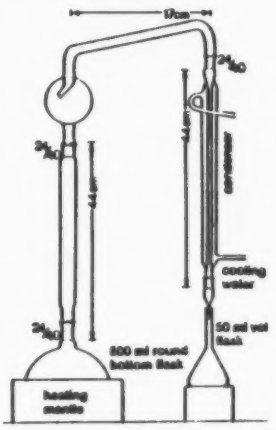


Figure 13.3 Vertical distillation assembly (18)

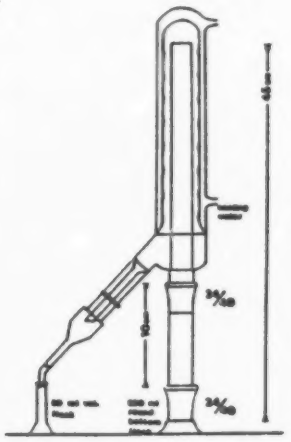


Figure 13.4 Integrated vertical distillation assembly with condenser/ concentrator (18)

REAGENTS

1. **Hydrochloric acid (4N):** To 500 ml distilled water, add (with caution) 250 ml concentrated HCl

2. **Propyl gallate**

3. **Disodium ethylenediamine tetraacetic acid (EDTA)**

4. **Anti-bumping granules,** BDH Chemicals

5. **TBA reagent:**

   a. Add 1.44 g 2-thiobarbituric acid and 50 ml distilled water to a 500 ml volumetric flask. Vigorously disperse with magnetic stirrer.

   b. Add glacial acetic acid until the flask is two-thirds full.

   c. Stir vigorously for ten minutes or until the 2-thiobarbituric acid is almost completely dissolved.

   d. Fill the flask to the mark with glacial acetic acid.

6. **Standard TEP solution:**

   a. Accurately weigh 0.22 g of standard 1,1,3,3-tetraethoxypropane (TEP) into a 100 ml volumetric flask and dilute to volume with distilled water.

   b. Pipette 10 ml of this solution into a 1 liter volumetric flask and dilute to volume with distilled water to produce a $1x10^{-4}$ M stock solution. Store refrigerated.

   c. A $1x10^{-5}$ M working solution is prepared by diluting 10 ml of the stock solution to 100 ml with distilled water.

7. **Standard buffer solutions** pH 1 and pH 2 (for Method B only).

8. **Antifoam B** Dow-Corning.

PROCEDURE

METHOD A - THIOBARBITURIC ACID REACTIVE SUBSTANCES (TBARS)

This procedure is applicable to mackerel and herring only. It must be tested for other species (see General Discussion).

a. Distillation of sample (TBARS)

1. Without thawing, transfer a 10 g portion of fish to the blender jar with 35 ml of distilled water and blend for 2 minutes or until sample is finely divided.

2. While blending, add 100 mg (approximately) each of propyl gallate and EDTA to a 500 ml round bottom flask. Add a few anti-bumping granules.

3. Quantitatively transfer sample homogenate to round bottom flask and add distilled water so that total weight of sample and water is 105 g.

4. Flush flask liberally with nitrogen and add 95 ml of 4N HCl.

5. Immediately connect to distillation apparatus (Figure 13.3 or 13.4) and collect 50 ml of distillate in a volumetric flask. Distillation should be complete in 35 minutes or less.

6. Rinse the still with methanol and then distilled water. All joints must be tight and should be wet during distillation.

7. Distillates may be refrigerated overnight if necessary.

b. Development of color

1. For standard curve accurately pipet aliquots of 0, 0.4, 0.8, 1.0, 1.2, 1.6 and 2.0 ml of working TEP standard solution ($1x10^{-5}$) into screw capped tubes. This is equivalent to 0,

0.004, 0.008, 0.01, 0.012, 0.016 and 0.02 u moles TEP respectively. Carefully add water to a total of 5 ml.

2. Pipet 5 ml of each sample distillate into screw cap test tubes. For strongly oxidized samples use less distillate and make up to 5 ml with distilled water.

3. Prepare a blank with 5 ml distilled water.

4. Add 5 ml TBARS reagent and tightly cap the tubes. Mix thoroughly (Vortex-mixer).

5. Heat the test tubes for 45 minutes in a vigorously boiling water bath. Cool in tap water.

6. Determine absorbance at 538 nm within one half hour of cooling. Spectrophotometer should be set to zero absorbance with the blank (0.0 ml TEP) in both "sample" and "reference" positions. Blank should be left in the "reference" position during all subsequent readings.

METHOD B - TARLADGIS THIOBARBITURIC ACID VALUE (TBA)

a. Distillation of sample (TBA)

1. Without thawing, transfer a 10 g portion of fish to the blender jar with 35 ml of distilled water and blend for 2 minutes or until sample is finely divided.

2. While blending, add 100 mg (approximately) each of propyl gallate and EDTA to a 500 ml round bottom flask. Add a few anti-bumping granules.

3. Transfer sample homogenate to 250 ml beaker and with distilled water rinsing, make up to 110 grams.

4. Adjust pH of the sample homogenate to pH 1.5 with 4N HCl. This pH should be maintained for at least 3 minutes. **N.B.** The pH meter must be carefully standardized with standard buffer solutions of pH 1 and pH 2 (see laboratory method 1, pH measurement).

5. **Alternatively,** if sample is homogeneous, blend 10 gram aliquots with 75 ml of water and determine the volume (mls) of 4N HCl required to bring pH to 1.5. Repeat about 4 times and calculate average HCl volume required for the neutralization.
Then add exactly that volume HCl to all samples. This avoids the incorporation of air during adjustment on the pH meter.

6. Transfer sample quantitatively to round bottom flask. Flush liberally with nitrogen and add 3 drops of antifoam B.

7. Continue as in step a.5 of method "A". Color development follows "b" of method "A".

CALCULATIONS

1. From the standards plot absorbance vs μ moles TEP as illustrated in Figure 13.5 below:

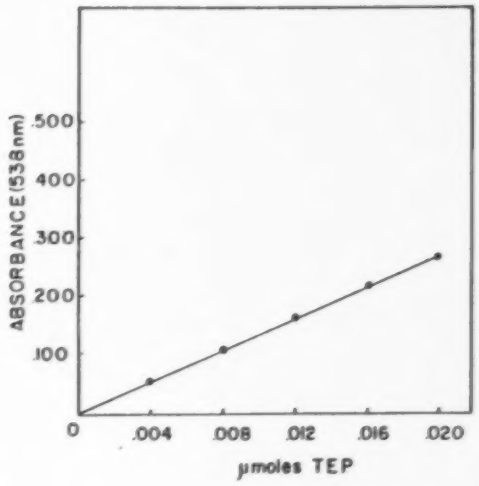


Figure 13.5 Typical TBA standard curve; Absorbance vs μ moles TEP in test tube.

2. For each sample determine μ moles TEP in tube (from standard curve).

3. Calculate TBA or TBARS as μ mole malonaldehyde or TBA reactive substances per kilogram fish from the formula:

$$TBA = \frac{T \times V_1 \times 1000}{V_2 \times W} \qquad (1)$$

where:

TBA = TBA or TBARS value in μ mole/kg fish
T = μ moles malonaldehyde (TEP) equivalent to absorbance of sample as determined from standard curve (Figure 13.5)
V_1 = volume (ml) of distillate collected, usually 50 ml
V_2 = volume (ml) of distillate aliquot withdrawn for analysis
W = weight of fish added to still, usually 10 g

N.B.: Rather than determing T from the graph directly, an equation can be determined for the line (Absorbance vs μ moles TEP) and equivalent μ moles standard solved algebraically.

EXAMPLE

From a 10 g (W) sample of mackerel, 50 ml (V_1) of distillate was collected in a TBARS analysis (Method A). With 5 ml (V_2) distillate an absorbance of 0.110 was obtained on color development. From the standard curve (similar to Figure 13.5) equivalent TEP in the test tube was determined to be 0.0086 μ moles (T). therefore TBARS value from formula 1 is:

$$TBA = \frac{0.0086 \times 50 \times 1000}{5 \times 10}$$

= 8.6 μ moles per kg fish

REFERENCES

1. LABUZA, T.P. 1971. Kinetics of lipid oxidation in foods. CRC Critical reviews in Food Technology. 2:355.

2. KE, P.J., R.G. Ackman, B.A. Linke, and D.M. Nash. 1977. Differential lipid oxidation in various parts of frozen mackerel. J. Food Tech. 12:37.

3. MELTON, S.L. 1983. Methodology for following lipid oxidation in muscle foods. Food Techn. July:105.

4. GRAY, J.I. 1978. Measurement of lipid oxidation: A review. J. Am. Oil Chemists' Soc. 55:539.

5. TARLADGIS, B.G. and B.M. Watts. 1960. Malonaldehyde production during the controlled oxidation of pure unsaturated fatty acids. J. Amer. Oil Chem. Soc. 37:403.

6. VISWANATHAN NAIR, P.G., P.D. Anthony and K. Gopakumar. 1979. Oxidative rancidity in the skin and muscle lipids of oil sardine (Sardinella longiceps). J. Food. Sci. Tech. 16:151.

7. HOLMAN, R.T. 1954. In Progress in the Chemistry of fats and other lipids. (R.T. Holman, ed.). Pergamon Press, London.

8. WYATT, C.J. and E.A. Day. 1963. Auto-oxidation of fish oils. II. Changes in the carbonyl distribution of autooxidizing salmon oils. J. Food Science. 28:305.

9. WILBUR, K.M., F. Bernheim and O.W. Shapiro. 1949. The thiobarbituric aicd reagent as a test for the oxidation of unsaturated fatty acids by various agents. Arch. Biochem. Biophys. 24:305.

10. TURNER, E.W., W.D. Paynter, E.J. Montie, M.W. Bessert, G.M. Struck and F.C. Olson. 1954. Use of the 2-thiobarbituric acid reagent to measure rancidity in frozen pork. J. Food Tech. July :326.

11. YU, T.C. and R.O. Sinnhuber. 1957. 2-thiobarbituric acid method for the measurement of rancidity in fishery products. J. Food Tech. February:104.

12. SIDWELL, C.G., H. Salwin, M. Benca and J.H. Mitchell Jr. 1954. The use of thiobarbituric acid as a measure of fat oxidation. J. Amer. Oil Chem. Soc. 31:603.

13. TARLADGIS, B.G., B.M. Watts and M.T. Younathan. 1960. A distillation method for the quantitative determination of malonaldehyde in rancid foods. J. Amer. Oil Chem. Soc. 37:44.

14. CASTELL, C.H. and G.A. Boyce. 1966. Erroneous thiobarbituric acid values in fish tissues caused by their normal content of free iron. J. Fish. Res. Bd. Canada, 23(10):1587.

15. CASTELL, C.H., B.A. Moore, P.N. Jangaard and W.A. Neal. 1966. Oxidative rancidity in Frozen Stored Cod Fillets. J. Fish. Res. Bd. Canada, 23(9):1385.

16. CASTELL, C.H., B. Moore and W. Neal. 1966. Cause and control of spurious thiobarbituric acid values from fish muscle in the presence of inorganic iron salts. J. Fish. Res. Bd. Canada, 23(5):737.

17. WOYEWODA, A.D. and P.J. Ke. 1979. Quality Assessment of fatty fish by the 2-thiobarbituric acid distillation method. Fish. Oceans Can., Tech. Branch. New Series Circular No. 63 (February).

18. ROBLES-MARTINEZ, C., E. Cervantes and P. J. Ke. 1982. Recommended method for testing the objective rancidity development in fish based on TBARS formation. Can. Technical Report of Fisheries & Aquatic Sciences No. 1089.

19. VYNCKE, W. 1970. Direct determination of the thiobarbituric acid value in trichloracetic acid extracts of fish as a measure of oxidative rancidity. Fette. Seifen, Anstrichmittel. 72:1084.

20. LEMARS, D.W. 1975. An improved TBA test for rancidity. New Series Circular No. 51. Halifax Laboratory, Fisheries and Marine Service, Canada.

21. SIU, G.M. and H.H. Draper. 1978. A survey of the malonaldehyde content of retail meats and fish. J. Food Sci. 43:1147.

22. KWON, T. and B.M. Watts. 1964. Malonaldehyde in aqueous solution and its role as a measure of lipid oxidation in foods. J. Food Sci. 29:294.

23. ASAKAWA, T. and S. Matsushita. 1979. Thiobarbituric Acid Test for Detecting Lipid Peroxides. Lipids, Vol. 14, No. 4:401.

24. KE, P.J., R.G. Ackman, and D.M. Nash. 1975. Proposed rancidity indexes for assessing the storage life of frozen mackerel. Environment Can., Fish. and Marine Serv., Hfx, N.S. New Series Circular No. 52. July 31, 1975.

25. BOTTA, J.R., J.T. Lauder, A.P. Downey, and W. Saint. 1983. Chemical and sensory assessment of nonspawning capelin (Mallotus villosus) subjected to long term storage. J. Food Sci. 48:1512.

26. LINDSAY, R.C., D.A. Stuiber, B. Stewart and V.C. Carlson. 1981. Evaluation of Burbot (Lota lota) acceptability for processing. Can. Inst. Food Sci. Technol. J. 14:196.

27. DENG, J.C., R.F. Matthews and C.M. Watson. 1977. Effect of chemical and physical treatments on rancidity development of frozen mullet (Mugil cephalus) fillets. J. Food Sci. 42:344.

28. KOLAKOWSKA, A. and J. Deutry. 1983. Some comments on the usefulness of 2-thiobarbituric acid (TBA) test for the evaluation of rancidity in frozen fish. Nahrung 27(1983) 5:513.

29. MALEKI, M. 1974. Validity of the 2-thiobarbituric acid test for the quantitative determination of rancidity in fats and oils. Fette. Seifen. Anstrichmittel 76:181.

30. KWON, T., D.B. Henzel and H.S. Olcott. 1965. Reactivity of malonaldehyde with food constituents. J. Food Sci. 30:808.

31. DAHLE, L.K., E.G. Hill and R.T. Holman. 1962. The thiobarbituric acid reaction and the autotoxins of polyunsaturated fatty acid methyl esters. Arch. Biochem. Biophys. 98:253.

32. BUTTKUS, H. and R.J. Bose. 1972. Amine-malonaldehyde condensation products and their relative color contribution in their thiobarbituric acid test. J. Amer. Oil Chem. Soc. (1972) 49:440.

33. CRAWFORD, D.L. 1967. Reaction of malonaldehyde with protein. J. Food Sci. 32:332.

34. TARLADGIS, B.G., A.M. Pearson and L.R. Dugan, Jr. 1962. The chemistry of the 2-thiobarbituric acid test for the determination of oxidative rancidity in foods. I. Some important side reactions. J. Amer. Oil Chem. Soc. 39:34.

35. RHEE, K.S. 1978. Minimization of further lipid peroxidation in the distillation 2-Thiobarbituric acid test of fish and meat. J. Food Sci. 43:1776.

36. MARCUSE, R. and Johansson. 1973. Studies on the TBA test for rancidity grading: II. TBA reactivity of different aldehyde classes. J. Amer. Oil Chem. Soc. 50:387.

37. WOYEWODA, A.D. and S.J. Shaw. 1984. Unpublished data.

38. YU, T.C. and R.O. Sinnhuber. 1964. Further observations on the 2-thiobarbituric acid method for the measurement of oxidative rancidity. J. Amer. Oil Chem. Soc. (48)8:549.

14. PEROXIDE VALUE (POV)

GENERAL DISCUSSION

Peroxides are the primary products of oxidation. However, since they are relatively short lived, their usefulness as oxidation indicators is limited to early stages of rancidity development. As oxidation proceeds peroxides break down to aldehydes (1) or combine with proteins (2).

Several procedures have been developed to measure peroxides. Most are based on the iodometric methods of Lea (3) and Wheeler (4) and involve measurement of iodine liberated from potassium iodide by peroxides (1) by titration (5,6), colorimetric (7,8) and coulometric (9) methods. Criticisms of these iodometric (9) procedures have included oxygen error (liberation of iodine from potassium iodide by atmospheric oxygen) and absorption of iodine by double bonds (10). Ferric thiocyanate (11,72), 2,6 dichlorophenolindophenol (72) and diphyenylcarbohydrazine (14) have also been used for peroxide reduction. The iodometric method adopted by the AOCS, although empirical and subject to variation, is still the method of choice by most laboratories.

Peroxide value changes in oil sardine (15, 16,17), mackerel (19,20,21,22,18), salmon oil (23), mackerel oil (24), salmon (25), herring (26) and mullet (27) have been reported under various conditions of fresh and frozen storage. The relationship of POV to flavour in oxidized foods depends on the type of muscle and processing (1). Tentative values for POV corresponding to edibility in mackerel have been reported by Ke et al. (18,22); POV of 2 and 12 meq/kg oil in mackerel meat and skin respectively was considered of borderline acceptability.

a. **Application:**

The peroxide value test (POV) is applicable for the measurement of early to intermediate stages of oxidation in fats, oils and lipids extracted from marine products.

b. **Principle**

The AOCS iodometric procedure (5) is based on the reduction of peroxides in acidic medium by potassium iodide. Liberated iodine is titrated with sodium thiosulfate with the aid of freshly prepared starch indicator. The reaction proceeds as follows:

$$ROOH + 2I^- + 2H^+ \longrightarrow ROH + H_2O + I_2$$
$$I_2 + 2S_2O_3^{--} \longrightarrow 2I + S_4O_6$$

c. **Precautions:**

1. Extracted lipid must be protected from oxygen, light and heat until analysis. During the titration oxygen must be excluded by a flow of nitrogen.

2. Difficulty with the endpoint will be encountered if starch indicator is not prepared fresh daily.

3. Visualization of starch endpoint depends on rapid magnetic stirring such that at each dropwise addition of $Na_2S_2O_3$ (before the endpoint) a white spot can be seen to move through the solution. At the endpoint this spot is not visible.

4. Thiosulfate solution (0.1N) must be standardized as described in Method A.

SAMPLE PREPARATION:

Blend freshly thawed sample (until just pliable) in a Cuisinart Food Processor with nitrogen flushing. Work quickly, do not expose sample to excess heat. Extract lipid by laboratory method 3B and store under nitrogen atmosphere. Although POV may increase slightly, lipid may be stored overnight if frozen.

APPARATUS

1. **Stopwatch**

2. **Magnetic stirrer**

3. **Glassware**
250 ml iodine flasks, glass stoppered, 125 ml Erlenmeyer flasks, pipets, (1 ml, 10 ml, 25 ml capacity), accurate 10 ml burette, burette (50 ml)

REAGENTS:

1. **Acetic acid/chloroform solution** - Mix 3 parts by volume (300 ml) of reagent grade glacial acetic acid with 2 parts by volume (200 ml) of reagent grade chloroform.

2. **Sodium thiosulfate, stock solution 0.1 N.** In 1 litre volumetric flask dissolve 24.8 g $Na_2S_2O_3 \cdot 5H_2O$ in recently boiled distilled water and make up to the mark. This solution is also available commercially.

3. **Sodium thiosulfate working solution, 0.01 N.** Accurately pipet 10 ml 0.1 N stock sodium thiosulfate solution into a 100 ml volumetric flask and bring to volume with recently boiled distilled water.

4. **Potassium iodide solution, 0.904 M.** Dissolve 15 g potassium iodide ACS, in distilled water and make to volume in a 100 ml volumetric flask.

5. **Saturated potassium iodide solution.** To 20 ml recently boiled distilled water in a 250 ml amber glass bottle add ACS grade KI until crystals remain undissolved. Warm slightly with tap water to aid dissolution. Store refrigerated away from light. This solution should be colourless and produce a blank of zero. To test, add 30 ml acetic acid/chloroform solution, 0.5 ml saturated KI and 2 drops starch indicator solution. If a blue color forms and requires more than 2 drops 0.01 N thiosulfate to dissipate, prepare a fresh solution of KI.

6. **Starch indicator solution.** Disperse 1 g starch in 10 ml cold distilled water and add to 100 ml vigorously boiling distilled water. Boil 1 minute. Cool before use. This solution must be prepared daily to ensure a sharp endpoint to the titration.

7. **Standard potassium dichromate solution,** 0.1 N. Dry 2 g ACS grade $K_2Cr_2O_7$ at 110°C overnight. Cool in desiccator. To a 100 ml volumetric flask add 0.490 g dried $K_2Cr_2O_7$ (accurately weighed) with 80 ml distilled water. Swirl to dissolve and make up to volume at 25°C.

8. **Hydrochloric acid,** ACS grade.

PROCEDURE

A. **Standardization of thiosulfate (0.1N)**

1. Pipet 25 ml aliquots of standard dichromate solution into 125 ml Erlenmeyer flasks.

2. With graduated cylinders add 5 ml hydrochloric acid and 10 ml KI **solution, 0.904 M. Swirl to mix.** Allow to stand 5 min.

3. Add 100 ml distilled water and titrate with 0.1 N thiosulfate until yellow colour has almost disappeared.

4. Add ca 0.5 ml starch indicator with pasteur pipette.

5. With stirring continue titration until blue colour has just disappeared.

6. Calculation of normality $Na_2S_2O_3$

$$N = \frac{2.5}{V_1} \qquad (1)$$

where:
N = normality $Na_2S_2O_3$
V_1 = volume $Na_2S_2O_3$ required for titration of standard

B. **Peroxide value determination**

1. Accurately weigh 2.5 to 5.0 g lipid into 250 ml ground glass stoppered iodine flasks previously flushed with nitrogen. Flush again and stopper.

2. Add 30 ml acetic acid/chloroform solution and swirl to dissolve lipid.

3. Add 1 ml saturated KI solution and swirl gently. Time for exactly 2 minutes with occasional swirling.

4. After 2 minutes add 100 ml water and approximately 1 ml starch solution (with pasteur pipette).

5. With vigorous magnetic stirring titrate with 0.01 N $Na_2S_2O_3$ until blue color disappears. Endpoint detection is aided if a white paper is place below titration vessel (see precautions).

6. A blank titration should be performed before each analysis to ensure freshness of KI.

CALCULATION

Peroxide value expressed as milliequivalents peroxide per 1000 g lipid may be calculated as:

$$POV = \frac{V_2 \times N \times 1000}{W} \qquad (2)$$

V_2= ml of thiosulfate for titration
N = normality of thiosulfate
W = weight of lipid in grams

EXAMPLE

A 3 g sample (W) herring oil required 4.32 ml (V_2) of 0.0131 N sodium thiosulfate (N) for peroxide value titration. Peroxide value from formula 2 is:

$$POV = \frac{4.32 \times 0.0131 \times 1000}{3.0}$$

$$= 18.9 \text{ m eq./1000 g oil}$$

REFERENCES

1. GRAY, J.I. 1978. Measurement of lipid oxidation: A review. J. Am. Oil Chemists' Soc. 55:539.

2. GARDNER, H.W. 1979. Lipid hydroperoxide reactivity with proteins and amino acids: A review. J. Agric. Food Chem. 27:220.

3. LEA, C.H. 1931. Royal Soc. London. 1088:175.

4. WHEELER, D.H. 1932. Oil Soap 9:89.

5. "Official and Tentative Methods of the American Oil Chemists' Society", Vol. 1, Third Edition, AOCS, Champaign, IL, 1971.

6. SULLY, B.D. 1961. "DFG-Einheitsmethode C-VIa", Wissenschaftl. Verlags gesellschaft m.b.H., Stuttgart, Germany.

7. HEATON, F.W. and N. Uri. 1958. Improved iodometric methods for the determination of lipid peroxides. J. Sci. Food Agric. 9:781.

8. SWOBODE, P.A.T. and C.H. Lea. 1958. Chem. Ind. 1090.

9. FIEDLER, V. 1974. J. Amer. Oil Chem. Soc. 51:101.

10. MEHUNBACHER, V.C. 1960. "The Analysis of Fats and Oils." Garrard Press, Champaign, Il.

11. STINE, C.M., H.A. Harland, S.T. Coulter and R. Jenness. 1954. J. Dairy Sci. 37:202.

12. LEA, C.H. 1952. J. Sci. Food Agric. 3:586.

13. LIPS, A., R.A. Chapman and W.D. McFarlane. 1943. Oil Soap 20:240.

14. HAMM, D.L., E.G. Hammond, V. Parvanah and H.E. Snyder. 1965. J. Amer. Oil Chem. Soc. 4:920.

15. VISWANATHAN NAIR, P.G., K. Gapakumar and M.R. Nair. 1976. Lipid hydrolysis in mackerel (Rastrellinger Kanagurta) during frozen storage. Fish. Technol. 13(2):111.

16. VISWANATHAN NAIR, P.G., P.D. Anthony and Gopakumar. 1979. Oxidative rancidity in the skin and muscle lipids of oil sardine (Sardinella longiceps). J. Food Sci. Tech. 16:151.

17. SRIKAR, L.N. and G.G. Hiremath. 1972. Fish preservation studies on changes during storage of oil sardine. J. Food Sci. and Tech. 9:191.

18. KE, P.J., R.G. Ackman, and D.M. Nash. 1975. Proposed rancidity indexes for assessing the storage life of frozen mackerel. Environment Can., Fish. and Marine Serv., Hfx, N.S. New Series Circular No. 52. July 31, 1975.

19. KE, P.J., R.G. Ackman, B.A. Linke, and D.M. Nash. 1977. Differential lipid oxidation in various parts of frozen mackerel. J. Food Tech. 12:37.

20. FARRAGUT, R.N. 1972. Effects of some antioxidants and EDTA on the development of rancidity in Spanish Mackerel (Scomberomorus maculatus) during frozen storage. NCAA Technical Report NMFS SSRF-650.

21. KE, P.J., B.A. Linke and R.G. Ackman. 1978. Acceleration of lipid oxidation in frozen mackerel fillet by pretreatment with microwave heating. J. Food Sci. 43:38.

22. KE, P.J., D.M. Nash and R.G. Ackman. 1976. Quality preservation in frozen mackerel. J. Inst. Can. Sci. Technol. 9:135.

23. WYATT, C.J. and E.A. Day. 1963. Autoxidation of fish oils. II. Changes in the carbonyl distribution of autooxidizing salmon oils. J. Food Sci. 28:305.

24. KE, P.J. and R.G. Ackman. 1976. Metal-catalyzed oxidation in mackerel skin and meat lipids. JAOCS 53:636-640.

25. BOYD, J.W., P.J. Schmidt, D.R. Idler and H.L.A. Tarr. 1957. Rancidity in stored fish. V. Can. Res. Bd. Progress Reports of the Pacific Coast Station, 108:21.

26. BILINSKI, E., R.E. Jonas and Y.C. Lau. 1978. Chill storage and development of rancidity in frozen Pacific herring, (Clupea harengus pallosi). J. Fish. Res. Board. Can. 35:473-477.

27. DENG, J.C., R.F. Matthews and C.M. Watson. 1977. Effect of chemical and physical treatments on rancidity development of frozen mullet (Mugil cephalus) fillets. J. Food Sci. 42:344.

15. CARBONYL VALUE (COV)

GENERAL DISCUSSION

During the course of oxidation, particularly in advanced stages or at elevated temperatures, peroxides are transformed into secondary products containing carbonyls (C=O) (1). The carbonyl value (COV) is therefore a measure of end products which are more stable than peroxides and which are perhaps the direct precursors to more volatile odorous compounds (2). Although attempts have been made at isolating or characterizing specific carbonyl compounds (3), total carbonyl determinations are based on either the oxime (4) or the hydrazone (5,1) methods.

The most popular is the latter (Henick, 5) procedure incorporating a reduction step for elimination of peroxide interference (6, 1). Carbonyl values (determined by various methods) have been reported for food commodities (7,8,9) although some investigators have concluded that the carbonyl value changes erratically in frozen muscle foods (10,11,12, 13). According to Mai and Kinsella (14), the carbonyl value should be used in combination with other tests such as the 2-thiobarbituric acid value to assess lipid oxidation in frozen fish since from their results the COV test alone appeared unsuitable for the purpose.

a. Application

The COV procedure is applicable to the measurement of oxidation in advanced stages occurring in fats and oils or lipids isolated from tissue or meal by laboratory method 3B described in this manual. It is particularly of value where peroxides have decomposed (2) such as in situations where high oxidation has occured at high temperatures (eg, cooking oils). The carbonyl value should be applied in combination with the 2-thiobarbituric acid test (14) of laboratory method 13.

b. Principle

The COV procedure of Henick (5) modified by Watanabe (1) is based on the condensation of the carbonyl group (-C=O) with 2, 4-dinitrophenylhydrazine in trichloroacetic acid to form red colored hydrazones. Extinction coefficients of these alkali salts are identical at 430 nm for both saturated and unsaturated straight chain monoaldehydes but are uniquely different at 435 and 460 nm respectively. Thus from the absorbance at these two wavelengths the relative proportion of saturated and unsaturated monoaldehydes can be estimated (15). Carbonyl value is expressed as absorbance per gram lipid determined at 440 nm (1).

c. Precautions

It is critical that reagents and solvents used in the procedure be certified pure or purified to remove impurities (1, 11). Also, since there is no fixed correlation between peroxide interference and level, peroxides must be destroyed according to the given procedure (6, 1).

APPARATUS

1. **Spectrophotometer** for use at 440 nm.
2. **Filter paper** #4 Whatman or equivalent.
3. **Water bath** set at 65°C.
4. **Glassware:** 125 ml separatory funnels, glass funnels, 200 ml beakers, 100 ml volumetric flasks, 50 ml volumetric flasks, graduated cylinders, 5 ml glass pipettes, pipetting bulb and 200 ml round bottom flasks with stoppers.

REAGENTS

1. **Carbonyl-free benzene:** Test suitability of reagent grade benzene by measuring absorbance of benzene against water at 430 nm. If absorbance is less than 0.35, then benzene is acceptable without purification. To purify, add 5 g 2,4 dinitrophenylhydrazine, and 1 g trichloroacetic acid to 1 litre benzene in a 2000 ml round bottom flask. Reflux for 1 hour then distill, discarding first 50 ml and last 100 ml. Store in amber bottle away from light.

2. **Carbonyl-free ethanol:** To 2 litres of 95% ethanol add 1 g $NaBH_4$, reflux for 2.5 hr and distill. Discard first 50 ml and last 100 ml. Store in amber bottle away from light.

3. **Carbonyl-free methanol:** To 1 litre methanol add 5 to 10 g aluminum granules and 8 to 10 g KOH. Reflux for 1 hour and distill as for carbonyl free ethanol.

4. **2,4 dinitrophenylhydrazine:** Recrystallize 2,4 dinitrophenylhydrazine by dissolving 5 g in a minimum amount of hot carbonyl-free methanol. Cool slowly and collect crystals. Recrystallization should be repeated.

5. **2,4-DNPH solution, 0.05%:** Dissolve 0.5 g purified 2,4-dinitrophenylhydrazine in 1 litre carbonyl-free benzene. This solution is stable for several months.

6. **TCA, 4.3% w/v:** Dissolve 43 g trichloroacetic acid (TCA) in carbonyl-free benzene in a 1 litre volumetric flask and make up to volume.

7. **KOH, 40% w/v:** With gentle shaking and heating, dissolve 8 g KOH in 200 ml carbonyl-free ethanol. This solution should be prepared daily.

8. **HCl,1.2 N:** To a 500 ml Erlenmeyer add 450 ml distilled water and 50 ml concentrated HCl (12 N).

9. **KCl (acidic) 20% w/v:** Dissolve 100 g KCl in 400 ml 1.2 N HCL in a graduated 500 ml Erlenmeyer. Make up to volume with 1.2 N HCl.

10. **KCl (aqueous) 30%:** In a graduated 500 ml Erlenmeyer dissolve 150 g KCl in approximately 375 ml distilled water and make up to volume.

11. **Stannous chloride solution (for 10 samples):** To a 150 ml beaker add 50 ml methanol, 50 ml benzene and 0.5g stannous chloride.

12. **Standard $K_2Cr_2O_7$:** To a 100 ml volumetric flask add 0.280 g KOH, 80 ml distilled water and 0.0303 g $K_2Cr_2O_7$ (ACS grade). Stir to dissolve and bring to volume with distilled water.

13. **Anhydrous Na_2SO_4:** granular.

14. **KOH (0.05M):** To a 100 ml volumetric flask add 0.280 g KOH, dissolve and bring to volume with distilled water.

SAMPLE PREPARATION

1. Weigh sufficient comminuted sample to yield approximately 1 g fat. Do not allow sample to warm and protect from oxidation by nitrogen flushing.

2. Proceed with lipid extraction according to laboratory method 3B. Collect dried chloroform extract in a 100 ml round bottom flask.

3. Evaporate chloroform by means of a rotary evaporator or with a stream of nitrogen (protect sample from heat and oxygen).

4. Crude fat content may be derived by weighing flask and fat as outlined in method 3B.

PROCEDURE

A. **Peroxide Destruction(27)**

1. Add 10 ml stannous chloride solution to each 100 ml round bottom flask containing recovered fat. Store at room temperature for 2 hours, shaking occasionally.

2. To each, add 40 ml of 20% KCl solution (acidic), gently swirl and transfer to 125 ml separatory funnels. Rinse each flask with a further 5 ml of KCl solution to ensure complete transfer of lipid.

3. Rinse flasks with 25 ml benzene and add to separatory funnels. Stopper, invert carefully to mix phases, and return funnels to facilitate phase separation.

4. From the top of each funnel carefully decant as much of benzene layer as possible into 100 ml beakers. Save the benzene. Add 25 ml of fresh benzene to each funnel and repeat inversion, separation, and decantation. Save the benzene.

5. Add a third aliquot of benzene to separatory funnels and invert to mix. After phase separation drain and discard each lower phase (KCl) taking care not to lose any benzene. Return collected benzene portions to respective separatory funnels rinsing beakers with ca 10 ml fresh benzene.

6. To each funnel add 25 ml aliquots of 30% KCl (aqueous), invert, allow separation, and discard bottom layer. Repeat 3 times for complete washing of benzene.

7. Slowly filter benzene into 100 ml volumetric flasks through glass funnels lined with #4 Whatman filter paper half filled with anhydrous granular sodium sulphate (to remove moisture). Rinse separatory funnels with fresh benzene and continue to wash sodium sulfate until volumetric flasks are filled to the mark.

B. Fat Concentration in Extract

1. Preweigh three disposable aluminum weighing dishes per extract to the nearest 0.001 g. Place dishes on an aluminum tray.

2. In a fume hood and with a rubber bulb, carefully pipette three 10 ml aliquots of each benzene solution into the aluminum weighing dishes (10 ml per dish). Allow the benzene to evaporate in the fume hood (dust free) until only a fat residue remains (ca 1-1.5 hr).

3. Transfer the dishes to a drying oven set at 110°C for 1 hour, cool to room temperature (no more than 15 min) and carefully weigh.

4. Calculation of fat content:

$$L = \frac{\text{weight of residue}}{\text{volume of extract}}$$

$$= \frac{(W_2 - W_1)}{V_1} \qquad (1)$$

Where: L = g lipid per ml benzene
W_2 = weight of dish + residue
W_1 = weight of empty dish
V_1 = volume of extract

C. Calibration of Spectrophotometer

1. Set spectrophotometer to 440nm.

2. With 0.05 M KOH in cuvettes placed in reference and sample compartments set absorbance to zero.

3. Remove cuvette from sample compartment and replace with standard $K_2Cr_2O_7$.

4. Record absorbance of $K_2Cr_2O_7$ (with 0.05 M KOH as reference) at 440 nm.

5. Calculate absorbance correction factor as:

$$F = \frac{\text{Published absorbance value}}{\text{determined absorbance value}}$$

$$= \frac{0.502}{A_1} \qquad (2)$$

Where:
F = absorbance correction factor
A_1 = absorbance of standard $K_2Cr_2O_7$

D. Carbonyl Color Development

1. Into each 50 ml volumetric flask pipet 3 ml TCA solution, 5 ml 2,4-DNPH solution and 5 ml benzene extract (containing fat). Substitute extract with 5 ml purified benzene for a blank determination.

2. Stopper the flasks (glass stoppers), swirl to mix reagents and heat in a 65°C water bath for 30 minutes.

3. Cool to room temperature with running water. This solution is stable for several hours.

4. To each flask pipet 10 ml KOH solution, (5% w/v) incubate at room temperature exactly 10 minutes and dilute to volume with carbonyl-free ethanol.

5. Incubate at room temperature for 10 minutes and measure absorbance of the solutions against the blank prepared in Step 1.

CALCULATION

Value for total carbonyl is expressed as:

$$COV = \frac{\text{Absorbance x correction factor}}{\text{lipid per ml x volume benzene per flask}}$$

$$= \frac{A_2 \times F}{L \times 5} \qquad (3)$$

Where:

COV = carbonyl value expressed in absorbance units per g lipid

A_2 = absorbance of 2,4-DNPH derivative measured at 440 nm

F = absorbance correction factor determined from formula above

L = fat content in g/ml from formula 1

EXAMPLE

1. Mackerel was prepared by filleting, removal of skin membrane and comminuting. A ten gram sample was used for fat extraction according to laboratory method 3B and yielded 1.10 g lipid after chloroform removal. Crude fat content was calculated as:

$$\frac{1.10 \text{ g}}{10 \text{ g}} \times 100 = 11\% \text{ lipid}$$

2. After a peroxide destruction and upon removal of 10 ml benzene extract for lipid determination, weight of dish and dish with residue were found to be 1.411 g (W_1) and 1.494 g (W_2) respectively. Lipid content per ml was calculated from formula 1 as:

$$L = \frac{1.494 - 1.411}{10}$$

$$= 0.0083 \text{ g per ml}$$

3. Absorbance of the standard $K_2Cr_2O_7$ was found to be 0.509 (A_1). Correction factor from formula 2 is:

$$F = \frac{0.502}{0.509}$$

$$= 0.986$$

4. During 2,4-DNPH derivatization, 5 ml extract was used and absorbance was 0.265 (A_3). Therefore carbonyl value from formula 3 is:

$$COV = \frac{0.265 \times 0.986}{0.0083 \times 5}$$

$$= 6.30 \text{ units per g lipid}$$

TBA value (TBARS) of the same sample according to laboratory method 13A was 44.0 umole/kg fish

REFERENCES

1. WATANABE, H. 1974. Determination of carbonyl value of fats and oils. Yukagaku (Oil Chemistry) 22:373-378. 1973. Fish. Res. Bd. of Can. Trans. Series No. 2889.

2. GRAY, J.I. 1978. Measurement of lipid oxidation: A review. J. Am. Oil Chemists' Soc. 55:539.

3. LEA, C.H. and P.A.T. Swoboda. 1962. J. Sci. Food Agric. 13:148.

4. BHALERAO, J.G., J.G. Endres and F.A. Kummerow. 1961. J. Am. Oil Chemists' Soc. 38:600.

5. HENICK, A.S., M.F. Benca, and J.R. Mitchell. 1954. Estimating carbonyl compounds in rancid fats and foods. J. Amer. Oil Chem. Soc. 31:88.

6. MIZUNO, G.R. and J.R. Chipault. 1965. Interference of peroxides with the determination of total carbonyls in autoxidized fats. J. Amer. Oil Chem. Soc. 42:839.

7. MOOKHERJEE, B.D. and S.S. Chang. 1963. Characterization of the carbonyl compounds in reverted soybean oil. J. Amer. Oil Chem. Soc. 40:232.

8. GADDIS, A.M., R. Ellis, J. Shamey and G.T. Currie. 1965. Carbonyls in oxidizing Fat. VII. A comparison of methods of isolating monocarbonyl compounds. J. Am. Oil Chem. Soc. 42:620.

9. WYATT, C.J. and E.A. Day. 1963. Autoxidation of fish oils. II. Changes in the carbonyl distribution of autooxidizing salmon oils. J. Food Science. 28:305.

10. CAPORASO, F., and J.D. Sink. 1978. Lipid-soluble carbonyl components of bovine adipose tissue. J. Food Sci. 43:1379.

11. DIMICK, P.S. and J.H. MacNeil. 1970. Poultry Products. 2. Storage time-temperature effects on carbonyl composition of cooked turkey and chicken skin fraction. J. Food Sci. 35:186.

12. DIMICK, P.S., J.H. MacNeil and L.P. Grunden. 1972. Poultry products quality. Carbonyl composition and organoleptic evaluation of mechanically deboned poultry meat. J. Food Sci. 37:544.

13. KELLER, J.D. and J.E. Kinsella. 1973. Phospholipid changes and lipid oxidation during cooking and frozen storage of ground beef. J. Food Science 38:1200.

14. MAI, J. and J. E. Kinsella. 1979. Changes in lipid composition of cooked minced carp (Cyprinus carpio) during frozen storage. J. Food Sci. 44:1619.

15. AURE, L., J. Ottesen, G. Finne and H. Klostad. 1967. Oxidative Rancidification of Marine Oils. Reports on Technology Research Concerning Norwegian Fish Industry, Vol. 5, No. 3.

16. FREE FATTY ACID (FFA)

GENERAL DISCUSSION

In frozen storage, lipids of fish skin and muscle tissues undergo lipolytic changes (enzymatic) resulting in an accumulation of free fatty acids (1). Free fatty acid (FFA) production varies with storage temperature, muscle type, species, fat content and season and has been often used as a quality index for fish and other food products (2, 3, 4). Lean fish appear more prone to FFA formation than fat fish (5). In mackerel FFA increases proportionately with fat content except for skin tissue where hydrolysis occurs more slowly (6).

FFA may be produced from either triglycerides or phospholipids and depending on muscle type, one or the other will be the dominant precursor; in dark muscle, triglycerides and in ordinary muscle, phospholipids are hydrolyzed preferentially to FFA (7, 8, 9). Accumulation of FFA has been reported for most marine species including cod (5) mackerel (8, 10, 3) tuna (7), herring and sardine (11, 9, 10), trout (1), salmon (12) and squid (13).

Analysis of FFA as first proposed by Ayers in 1956 has undergone several modifications (14). The American Oil Chemists' Society standard FFA procedure (15) involves aqueous NaOH titration of lipid dissolved in ethanol with pheholphthalein indicator. The method has several drawbacks: a turbid solution must be titrated; carotenoids, pigments and oxidation products produce interfering color changes; the indicator endpoint fades; and the procedure is subject to operator error at very low or very high FFA (16, 17, 18). Suggested alternatives have included the use of potentiometric non-aqueous titrations (19, 20), enzymatic or chemical reaction with colorimetric determination (21, 22, 23, 24, 25, 26), gas chromatographic analysis (27), and monophasic/aqueous titration (28, 16).

The latter method utilizing a ternary mixture of chloroform, methanol, isopropanol in the proportions 2:1:2 completely solubilizes the fats and allows a sufficient amount of aqueous NaOH to be added for the titration before the cloud point is reached (turbidity). The adoption of metacresol purple indicator facilitates a very sharp end point and high reproducibility.

Two methods (A and B) are presented; the first is applicable to solid materials (flesh or meal) and involves preliminary separation of lipid by a method modified from the Bligh and Dyer procedure (29), while the second applies to lipid or oil samples.

a. Application

The free fatty acid titration is applicable to all fats, oils and marine fish which have not been acid treated during processing (e.g. pickling). Method B outlines a titration method for oil or purified lipid while method A applies to flesh or meal. The latter method (A) incorporates a step for estimation of fat content in the original flesh.

b. Principle

The FFA test (28, 16) is a sodium hydroxide titration of free carboxyllic acid groups present in marine lipid. Oil may be used directly or recovered in a chloroform/methanol extraction. With a final chloroform, methanol, isopropanol ratio of 2:1:2, aqueous NaOH is added until an endpoint is reached indicated by a color change of added meta-cresol purple. FFA is calculated as μ mole per gram oil (FFAF), tissue (FFAT) or alternately assumed to have a molecular weight of oleic acid and reported as percent by weight of oil (FFA).

c. Precautions

1. FFA increases with time in storage and temperature. Samples stored for analyses must be maintained at low temperatures (-35 to -40°C) and for short time periods only.

2. Generally 10 g may be used for samples up to 15% fat with 70 to 82% moisture.

With materials **low** in **moisture** (or high in fat) sample size should be decreased to avoid filtration and extraction problems, especially for salted products. When dealing with such material extra water should not be added during the blending step (method A; part "b", step 1); this action may promote emulsion formation.

3. Extreme agitation of flasks contents after water addition (method A, part "b", step 4) should be avoided to prevent emulsion formation. If samples are prone to emulsion formation (e.g. shellfish), water may be poured directly to the separatory funnel followed by gentle addition of chloroform/methanol extract. Emulsions sometimes separate without aid after longer settling times (15 to 24 hr) at room temperatures.

4. Overnight separation of chloroform/methanol/water extract is generally advised (method A, part "b", step 4). Premature removal of the chloroform phase may result in lower fat content which will affect FFAT (tissue) value. FFA(% oleic) on a lipid basis will not generally be affected by premature separation.

5. Meta-cresol purple is soluble in water only with the aid of NaOH. However, the amount of base added must not be excessive otherwise the blank titration will be affected. A blank of 0.05-0.1ml is considered satisfactory; dilute HCl or NaOH may be added to the indicator to achieve the desired blank value.

SAMPLE PREPARATION

FFA formation is enzymatic and therefore time and temperature dependent; samples must be protected from change during storage. Marine products should be comminuted and 10 g samples withdrawn for analysis. With dry or very high fat samples, smaller quantities should be used; e.g. for fish meal or salt fish use 1 to 2 g. If samples cannot be analyzed immediately they should be stored at -35 to -40°C. Oil or lipid may be analyzed directly according to method B.

APPARATUS

1. **Small blender** preferably with a glass jar, eg. Virtis Co. (Gardiner, N.Y.)

2. **Aluminum drying dishes,** disposable

3. **Filter paper** Whatman #1 and #4, 18.5 cm; Watman #4, 9 cm

4. **Drying oven** at 103°C

5. **Glassware:** 125 and 250 ml Erlenmeyer flasks, 250 ml separatory funnels, 10 ml micro-burette, 9 cm Buchner funnel assembly, 100 ml volumetric flasks weighing bottle, watch glass, 50 ml buret.

REAGENTS

1. **Sodium hydroxide (0.05N):** To 1 liter distilled water add 2.0 g NaOH. Pre-standardized NaOH is available from supply companies. Otherwise, NaOH should be standardized against potassium acid phthalate as decribed in method A, part "a".

2. **Aqueous meta-cresol purple indicator (0.5%):** Dissolve 0.5g m-cresol purple indicator in 26 ml of 0.05N NaOH. Make volume up to 100 ml with distilled water. Test indicator by adding 5 drops to a mixture of 50 ml chloroform, 25 ml methanol, and 50 ml 2-propanol in a 125 ml Erlenmeyer flask. A yellow solution will result which should change to violet upon addition of no more than 4 drops of 0.05 N NaOH. Adjust indicator with weak NaOH or HCl solution so that this criterion is met.

3. **Methanol** ACS grade

4. **Chloroform** ACS grade

5. **2-propanol (isopropanol)** ACS grade

6. **Anhydrous sodium sulfate** granular, ACS

7. **Potassium acid phthalate,** ACS analytical

grade 5-10 g dried overnight at 103°C.

8. **Phenolphthalein indicator:** dissolve 1 g phenolphthalein in 70 ml ethanol and with stirring add 30 ml distilled water.

9. **Chloroform/isopropanol/methanol solution (2:2:1):** In a 1 litre Erlenmeyer flask mix 200 ml chloroform, 200 ml isopropyl alcohol and 100 ml methanol.

PROCEDURE

METHOD A: Free Fatty Acid for Tissue

a. **Standardization of 0.05 N NaOH**

1. Into each of three clean 250 ml Erlenmeyer flasks, weigh 0.30 g of dried potassium acid phthalate. Add 50 ml of distilled water (to dissolve) and 3 drops phenolphthalein indicator to each flask.

2. Fill a 50 ml buret with sodium hydroxide solution (ca 0.05 N).

3. Titrate each potassium acid phthalate sample to the endpoint of the phenolphthalein indicator (first perceptible but permanent pink color) with 0.05 N NaOH.

4. Record the position of the meniscus on the buret and the volume of NaOH used.

5. Calculate NaOH normality as:

$$N_1 = \frac{W_1 \times 1000}{204.22 \times V_1} \qquad (1)$$

where: N_1 = normality NaOH
V_1 = volume (ml) NaOH used for titration
W_1 = weight (g) potassium acid phthalate in flask

6. Repeat for each of the remaining phthalate samples and calculate average normality of the NaOH.

b. **Determination of free fatty acid (tissue sample)**

1. To small blender jar, add 10 g sample, 50 ml chloroform and 50 ml methanol. Blend 1 minute until finely divided.

2. Filter on Buchner funnel through Whatman #4 filter paper and rinse with small amount of chloroform.

3. Add 45 ml distilled water to filtrate to achieve a final chloroform/methanol/water ratio of 1:1:1, swirl gently, and transfer to 250 ml separatory funnel. Rinse flask with chloroform, add washings to separatory funnel, stopper and leave 2 or 3 hours (or preferably overnight) at room temperature.

4. After equilibrium, using a regular funnel, slowly filter the lower chloroform layer from the separatory funnel through a double 18.5 cm filter paper (#4 Whatman inside and #1 outside) half filled with anhydrous sodium sulfate into a 100 ml volumetric flask. Rinse with chloroform but do not exceed mark of volumetric. Fill to mark with chloroform.

5. Pipet three 10 ml aliquots of chloroform filtrate into pre-weighed aluminum drying dishes and allow solvent from dish to evaporate in fume hood. When completely evaporated, place dish in a drying oven at 103°C for 1 hour, cool and weigh to the nearest 0.001 g.

6. Transfer remaining volumetric solution to 250 ml Erlenmeyer flask. Rinse with 10 ml chloroform.

7. Add 70 ml 2-propanol, 35 ml methanol, and 8 drops meta-cresol purple indicator.

8. Titrate to violet endpoint with 0.05 N NaOH.

9. A blank containing all reagents except the sample should be titrated. If more than 0.2 ml of titrant is required,

acidity of the indicator should be adjusted.

METHOD B: Free Fatty Acid in Lipid or Oil Sample

1. Extract lipid (as in laboratory method 38). If sample is an oil, use directly.
2. Accurately weigh 1 g lipid to nearest 0.001 g into a 125 ml Erlenmeyer flask.
3. Add 75 ml of chloroform/isopropyl alcohol/methanol solution and swirl to dissolve lipid.
4. Add 4-5 drops of meta-cresol purple indicator.
5. Titrate to violet endpoint with standard 0.05 N NaOH.
6. A blank containing all reagents except the sample should be titrated. If more than 0.2 ml of titrant is required, acidity of the indicator should be adjusted.

CALCULATIONS

A. From Method A

1. Fat content of the sample analyzed according to method A may be determined from the formula:

$$F = \frac{(D_2 - D_1) \times V_3}{V_2 \times W_2} \times 100 \qquad (2)$$

Where:

D_1 = weight empty drying dish (grams)
D_2 = weight of drying dish with fat residue (grams)
F = fat content in percent
V_2 = volume (ml) of aliquot withdrawn to aluminum dish
V_3 = volume (ml) of volumetric
W_2 = weight (g) of sample blended in analysis

2. Free fatty acid content determined from method A may be expressed in three ways:

a. On a tissue weight basis as **μ mole per gram tissue** from the formula:

$$FFAT = \frac{1000 \times N_1 \times (V_4 - V_5)}{W_2 - \frac{V_2 \times W_2 \times P}{V_3}} \qquad (3)$$

b. On a fat basis as **μ mole free fatty acid per gram fat** from the formula:

$$FFAF = \frac{FFAT}{F} \times 100 \qquad (4)$$

c. On a fat basis expressed as **percent free fatty acid (as oleic)** from the formula:

$$FFA = \frac{FFAT \times 2.82}{F} \qquad (5)$$

where:
FFA = free fatty acid of lipid as percent oleic acid
FFAF = free fatty acid of lipid as μ mole per gram (fat)
FFAT = free fatty acid of tissue as μ mole per gram (tissue)
P = number of aliquots of chloroform filtrate withdrawn from volumetric flask
V_4 = ml titrant (NaOH) used for sample
V_5 = ml titrant (NaOH) used for blank
F, V_1, V_2, V_3 and N_1 are as defined above

B. From Method B

Free fatty acid determined on lipid according to method B may be expressed in two ways:

a. On a lipid basis as μ mole per gram (fat) from the formula:

$$FFAF = \frac{1000 \times N_1 \times (V_4 - V_5)}{W_3 \times F} \qquad (6)$$

b. On a fat basis as percent free fatty acid (as oleic) from the formula:

$$FFA = \frac{N_1 \times (V_4 - V_5) \times 28.2}{W_3} \qquad (7)$$

where:
W_3 = weight (g) of lipid used for the analysis
F, N_1, V_4 and V_5 have been defined above

EXAMPLE

1. A 5 g sample (W_2) of squid liver was analyzed according to method A and the extract collected in a 100 ml (V_3) volumetric flask. After bringing to volume, three (P) 10 ml (V_2) portions of the $CHCl_3$ solution were withdrawn for fat analysis. Each empty dish weighed 1.447 (D_1) g.

2. In the titration 0.04 (V_5) and 8.52 ml (V_4) of 0.049 N NaOH (N_1) were required for the blank and sample, respectively.

3. After drying the three dishes containing fat weighed 1.590, 1.595 and 1.592 g, i.e. three values for D_2.

4. From formula (2) % fat may be calculated from the first value of D2 as :

$$\frac{(1.590 - 1.447) \times 100}{10 \times 5} \times 100 = 28.6\%$$

5. Free fatty acid (FFAT) as μ mole per gram sample from formula (3) is:

$$\frac{1000 \times 0.049 \times (8.52 - 0.04)}{5 - \frac{(10 \times 5 \times 3)}{100}}$$

$= 118.7\ \mu$ mole g^{-1} tissue

6. Free fatty acid (FFAF) as μ moles per gram fat from formula (4) is:

$$\frac{118.7 \times 100}{28.6} = 415.0\ \mu \text{ mole } g^{-1} \text{ fat}$$

7. Free fatty acid (FFA) percent as oleic acid from formula 5 is:

$$\frac{118.7 \times 2.82}{28.6} = 11.7\%$$

8. Lipid from another sample of squid liver was recovered according to laboratory method 3 B. A fat sample of 1.03 g (W_3) was weighed into a 125 ml enlenmeyer, solvents were added according to method B above and the mixture titrated with 0.049N NaOH (N_1). Blank was 0.04 ml (V_5) and volume of titrant used 8.56 ml (V_4). FFA percent as oleic may be calculated directly from formula (7) as:

$$\frac{0.049 \times (8.56 - 0.04) \times 28.2}{1.03}$$

$= 11.4\%$

REFERENCES

1. MAZEAUD, F. and E. Bilinski, 1976. Free fatty acids and the onset of rancidity in Rainbow trout (salmo gairdneri) flesh. Effect of phospholipen A. J. Fish. Res. Board Can. 33:1297.

2. DYER, W. J., 1968. In, Low Temperature Biology of Food stuffs, Pergamon Press, N.Y. pp. 429-447.

3. KE, P. J., R. G. Ackman, B. A. Linke and D. M. Nash, 1977. Differential lipid oxidation in various parts of frozen mackerel. J. Food Technol. 12:37.

4. EDWARDS-WEBB, J. D., 1975. J. Sci. Food Agric.

5. DYER, W. J. and D. I. Fraser, 1959. Proteins in fish muscle. 13 Lipids hydrolysis. J. Fish Res. Bd. Canada 16:43.

6. KE, P.J. and R.G. Ackman. 1976. Metal-catalyzed oxidation in mackerel skin and meat lipids. JAOCS 53:636-640.

7. TSUKUDA, N., 1976. Changes in the lipids of frozen fish. Bull. Tokai Reg. Fish. Lab 84:31.

8. VIVIANI, R., A. R. Borgatti, L. Mancini, and P. Cortesi, 1967. Changes in muscular lipids of the mackerel (Scombrus L.) during storage. Atti. Della Soc. Ital. Sci Vet. 21:706.

9. ADDISON, R. F., R. G. Ackman and J. Hingley. Free fatty acids of herring oils: Possible derivation of both phospholipids and triglycerides in fresh herring. J. Fish. Res. Bd. Canada 26:1577.

10. NAIR, P. G. V., K. Gopakumar, and M. R. Nair, 1976. Lipid hydrolysis in mackerel (Rastrellinger kamagrute) during frozen storage. Fish Technol. 13:111.

11. VISWANATHAN NAIR, P.G., P.D. Anthony and K. Gopakumar. 1979. Oxidative rancidity in the skin and muscle lipids of oil sardine (Sardinella longiceps). J. Food. Sci. Tech. 16:151.

12. BOTTA, J. R., J. F. Richards and N. Tomlinson, 1973. Thiobarbituric acid value, total long chain free fatty acids and flavour of Pacific halibut (Hippoglossus stenolepes) and chinook salmon (Oncorhynchus tshawytscha) frozen at sea. J. Fish Res. Board Canada 30:63.

13. WOYEWODA, A.D. and P.J. Ke. 1980. Laboratory Quality Assessment of Canadian Atlantic Squid (Illex illecebrosus)Department of Fisheries and Oceans, Halifax, Technical Report No. 902.

14. AYERS, C. W., 1956. Anal Chim Acta 15:77.

15. AMERICAN OIL CHEMISTS SOCIETY, Official and Tentative Methods of AOCS, 3rd edn, 1974, Champaign, Ill., U.S.A., Ca. 52-40.

16. KE, P. J., A. D. Woyewoda, L. W. Regier, and R. G. Ackman, 1976. An improved titrimetric method for determination of free fatty acid in fish oils. Fisheries and Environment Canada, Halifax laboratory, New Series Circular No. 61.

17. HOROWITZ, W., 1976. Methods of analysis approved by the Codex Alimenatarius Commission. I. Acid Value. J. Assoc. offic. Anal. chem. 59:658.

18. NERING, B and K. Modzelewska, 1975. From Tluscze jadalne 19:129. Fisheries and Marine Service, Halifax, Translation Series No. 3783.

19. INTERNATIONAL ORGANIZATION OF STANDARDIZATION, Technical Committee ISO/TC34-Agricultural Food Products Sub-Committee 11-Animal and vegetable Fats and oils, 1975. ISO/TC 34/SC 11.

20. BLIGH, E. G. and M. A. Scott, 1966. Lipids of cod muscle and the effect of frozen storage. J. Fish Res. Board Can. 23:1025.

21. BAKER, D., 1964. A Colorimetric method for determining free fatty acids in vegetable oils. J. Am Oil Chem Soc. 41:21.

22. TAKAHASHI, K. F., and C. Hayashi, 1975. Rinsho Kagaku 4:179.

23. KOOPS, J. and Klomp, H., 1977. Neth. Milk Dairy J. 31:56.

24. LOWRY, R. R. and Tinsley, I. J., 1976. J. Am. Oil Chem. Soc. 53:470.

25. CARRUTHERS, M. and D. A. B. Young, 1973. Clin. Chim Acta 49:341.

26. MOSINGER, F., 1965. Photometric adaptation of Dole's microdetermination of free fatty acids. J. Lipid Res. 6:157.

27. COCHRANE, G. C., 1975. A review of the analysis of free fatty acids (C_2-C_6). Journal of Chromatographic Science 13:440.

28. KE, P. J. and A. D. Woyewoda, 1978. A titrimetric method for determination of free fatty acids in tissues and lipids with ternary solvents and m-cresol purple indicator. Anal. Chim. Acta 99:387.

29. BLIGH, E.G. and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37:911.

17. EXTRACTABLE PROTEIN NITROGEN (EPN)

GENERAL DISCUSSION

Salt solubility has often been used as a criterion for protein classification (1). Reay and co-workers solubilized 85% of the available protein in haddock muscle with a 7% lithium chloride solution but noted that recovery was significantly reduced during frozen storage (2, 3, 4). Using cod muscle, Dyer et al. (5) optimized conditions for maximum salt soluble protein extraction. Requirements were 3 to 5% salt, pH of 7.0 to 9.0, a 1 to 20 ratio of fish to extractant, temperature not exceeding 5°C, protection from foaming, and most critically, sufficiently fine subdivision of the muscle fibrils. A recovery of up to 95% of the total protein in cod muscle was obtained by this method. $NaCl/NaHCO_3$ levels reported in the literature for successful EPN extractions have been 5%/0.02 M (6), 3.51%/0.003M (7, 8) and 5%/0.003 M (9, 10, 11). In each case the 3 to 5% NaCl and pH 7.0-7.5 ranges have been maintained.

Decrease of salt extractable protein during frozen storage especially in the gadoids, Alaska pollock and red hake (9), parallels the increased perceived toughness of the fish after cooking (12). The phenomenon appears to be caused by protein changes due to formaldehyde enzymatically produced during frozen storage (9, 13, 14, 15), transition metals (16) and other yet unexplainable factors (8). Analysis of extractable protein nitrogen has been used to study frozen storage changes in several species (10, 11, 9, 8, 17, 3, 13, 18, 19, 20) in an effort to become more familiar with causes and implications of the associated textural degradation.

a. Application

The extractable protein or "salt extractable" nitrogen test is applicable to the measurement of denaturative protein changes occurring during frozen storage. It is especially useful for determining changes in the gadoids (cod, pollock, hake) where salt extractability of the protein is accelerated by the enzymatic production of formaldehyde from trimethylamine oxide (9).

b. Principle

At a temperature below 5°C, intracellular protein is solublized in a 5% NaCl solution buffered with 0.003 M $NaHCO_3$. Maximum solubilization requires fine subdivision of the muscle fibrils through the use of a Waring blender equipped with a baffle plate (Figure 17.4) to prevent foaming and a modified bearing assembly (Figure 17.1) to eliminate air intake from the bottom of the jar (6).

After centrifugation, protein concentration of the filtrate is determined by a biuret procedure (21) simplified by Snow (22) combining cupric sulfate with sodium hydroxide as a single reagent. In the method an aliquot of protein solution containing 0.1 to 1.2 mg protein nitrogen is diluted to 5 ml with distilled water and reacted with the buiret reagent for 25 minutes to produce a violet pigment measureable at 540 nm. The reagent reacts with polypeptides having three or more amino acid components and with the amino acids histidine, serine and threonine. Albumin proteins produce a slightly stronger biuret color than muscle globulins (22). Ammonia interferes with the analysis by producing a deep blue cupric ammonium complex.

Bovine serum albumin, checked by Kjeldahl protein analysis, laboratory method 4, is used for standardization of the biuret determination. Alternately protein nitrogen may be determined in aliquots of salt extracted protein by both biuret and Kjeldahl methods to standardize the methods.

In cases where glycogen and fat produce turbidity (eg. scallops or fatty fish), procedures developed by Umemoto (23) incorporating a blank with NaOH/glycerine and Dingle (5) using NaOH should be applied. The latter will be adopted for the method which follows.

c. **Precautions**

1. Incorporation of air during the blending will cause foaming and protein recovery may be reduced due to protein denaturation. A baffle (Figure 17.4) must be used and the blending blade assembly modified by insertion of the "O" ring (Figure 17.1).

2. Temperature of the solution during blending must not rise above 5°C. Sample and jars should be kept cold and the entire extraction procedure may be carried out in a cold room.

3. Where turbidity is present during protein measurement from glycogen or lipid (scallops), the modified method (23) should be applied.

4. Measure color between 25 and 45 minutes after addition of the biuret reagent. After 45 minutes the solution may become cloudy (22).

APPARATUS

1. **Spectrophotometer,** set at 540 nm.

2. **Vortex mixer**

3. **Glassware:** 500 ml graduated cylinder, volumetric pipettes, 18x150 mm test tubes, 50 and 1000 ml volumetric flasks, 600 ml beakers, 50 ml beakers.

4. **Plastic bottles,** 60 ml with screw caps.

5. **Refrigerated centrifuge,** or centrifuge in a cold room, with 50 ml tubes

6. **Waring blender base,** preferably heavy duty model 840 watts.

7. **Blender jar,** 500 ml with "O" ring inserted in "oilite" bearing of blade assembly as shown in figure 17.1. The bronze "oilite" bearing should be pressed out of blade assembly, a groove machined, the unit lubricated thoroughly and reinstalled in the bearing holder assembly.

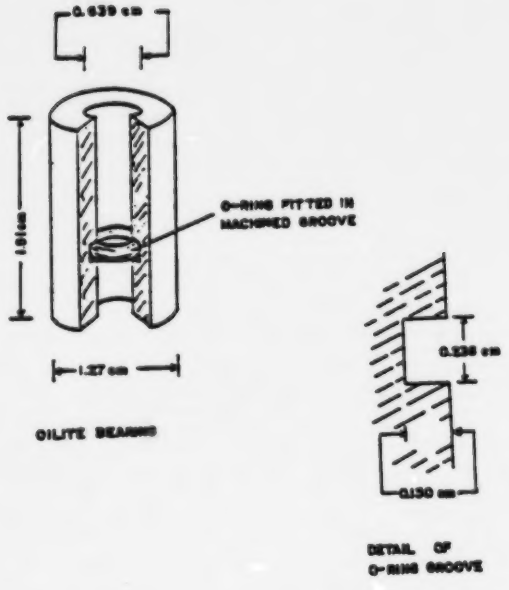


Figure 17.1 Insertion of "O" ring into oilite bearing of blade assembly.

For re-assembly, i.e. insertion of the socket head drive shaft, a "pilot tool" shown in Figure 17.2 below should be manufactured and used to prevent the sharp edges of the drive shaft cutting the "O" ring.

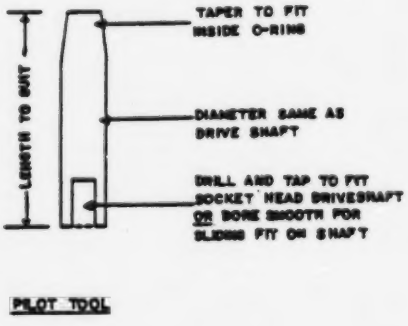

TAPER TO FIT INSIDE O-RING
DIAMETER SAME AS DRIVE SHAFT
DRILL AND TAP TO FIT SOCKET HEAD DRIVESHAFT OR BORE SMOOTH FOR SLIDING FIT ON SHAFT
LENGTH TO SUIT
PILOT TOOL

Figure 17.2 Pilot tool for insertion of socket head drive shaft

After insertion of the lubricated "O" ring into grove, place a fibre washer on the socket head drive shaft and the pilot

tool over threaded end as shown in Figure 17.3. Lubricate and slide assembly into the bearing holder assembly and through the O-ring. Remove the pilot tool and complete assembly.

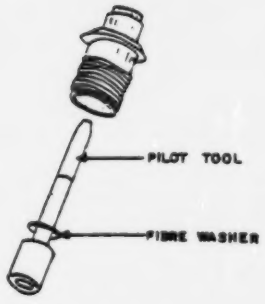


Figure 17.3 Application of pilot tool to blade assembly

3. **Baffle plate,** plexiglass to fit into blender jar about 1.5 cm below liquid level. See Figures 17.4 below.

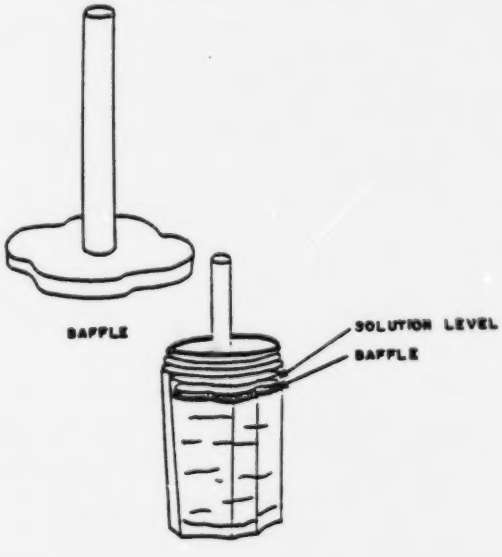


Figure 17.4 Plexiglass baffle, removed and in blender jar (not to scale).

REAGENTS

1. **Extracting buffer,** 5% NaCl in 0.003M $NaHCO_3$: in a 1000 ml volumetric flask dissolve 0.252 g $NaHCO_3$ and 50 g NaCl, ACS grade and fill to the mark with distilled water. Store refrigerated. This solution must be placed in a freezer before use until ice crystals just appear.

2. **Biuret reagent**

   a. In a 2000 ml graduated beaker add 400 ml distilled water and 180 g NaOH, ACS grade. Stir to dissolve and cool.

   b. Dissolve 2.0 g $CuSO_4 \cdot 5H_2O$ in 400 ml distilled water in a 600 ml beaker.

   c. Slowly and with vigorous stirring add the dissolved $CuSO_4 \cdot 5H_2O$ to the dissolved NaOH in the 2000 ml beaker. Too rapid addition will produce a precipitate of Cu_2O. Make up to 1 liter (in beaker) with distilled water.

3. **Turbidity reagent.** To a 1000 ml volumetric flask add 400 ml distilled water and 180 g NaOH. Stir to dissolve and cool. Make up to volume with distilled water.

4. **Bovine serum albumen (BSA),** 0.30%. Accurately weigh 0.075 g desiccated BSA into a 25 ml beaker. Add 10 ml distilled water to partially dissolve, transfer quantitatively to a 25 ml volumetric flask and carefully make up to the mark with distilled water.

SAMPLE PREPARATION

1. With a knife dice a representative sample of frozen fish muscle into small cubes (without thawing).

2. Weigh 22.0 g portions into sample cups and store frozen.

PROCEDURE

A. Extraction (5, 7, 24)

1. Store 430 ml portions of extracting buffer in the freezer until ice crystals are formed (ca -2.4°C). The blender jar should be packed in ice to cool before analysis begins.

2. To the blender jar add preweighed frozen sample and a 430 ml aliquot of chilled buffer.

3. Insert baffle into the blender jar ensuring that no air bubbles are trapped below (Figure 17.4).

4. Blend for ca 1 minute. With a glass rod or spatula scape tissue from blades, reposition baffle and blend 1 more minute. Check blades again, insert baffle and blend for 0.5 minutes. For unfrozen samples the 2.5 minute blending may be reduced to 2.0 minutes.

5. Pour extract into a 600 ml beaker. If ice crystals remain, allow these to melt before the next step.

6. Pour portions into centrifuge tubes and centrifuge for 30 minutes at 20,000 x g, ie. 13,000 rpm.

7. Carefully decant a portion of the supernatant into 50 ml beakers, or into 60 ml plastic bottles. Samples may be analyzed immediately, stored in the refrigerator for 1 day or alternately frozen at -30°C until analysis. If frozen or stored, samples should be well stirred to ensure homogeneity before withdrawal of aliquots for protein analysis.

B. Protein Analysis (Biuret, 22, 7)

1. For a standard curve add 0.5, 1, 2, 3, 4, and 5 ml portions of standard BSA solution to test tubes and make up to 5 ml with distilled water. Purity of BSA may be checked by Kjeldahl protein determination, laboratory method 4. Alternately a portion of salt extracted fish muscle protein solution can be analysed by Kjeldahl and biuret (this section) to establish standardization.

2. Prepare two blanks each with 5 ml of distilled water.

3. Accurately pipet aliquots of extracts (supernatants) containing 0.1 to 1.0 mg protein nitrogen (0.6 to 6.0 mg protein) into test tubes. Make up to 5 ml with distilled water. For freshly frozen cod or haddock 1 ml of extract (supernatant) should be used; for longer frozen material a larger volume may be required. If extracts are turbid prepare extra aliquots for turbidity correction.

4. To each tube, one blank included, pipet 5 ml of biuret reagent and stir thoroughly on the vortex mixer (caution should be exercised in mixing since solution is caustic). Allow to stand for 20 minutes at room temperature for full development of violet colour.

5. For turbidity correction, to the extra aliquots (prepared in Step 3) and remaining blank add 5 ml turbidity reagent and mix thoroughly on the vortex mixer.

6. Set spectrophotometer (at 540 nm), to zero absorbance with turbidity blank in both reference and sample cells. Measure absorbance of samples prepared in step 5 against turbidity blank.

7. Re-zero spectrophotometer with biuret blank (step 4) and measure absorbance of reacted standards and extracts against biuret blank. If biuret absorbance of extract minus corresponding turbidity absorbance is greater than highest absorbance obtained for any standard, the analysis should be repeated with a smaller volume of extract.

8. For accuracy in the calculations moisture of the fish samples should be determined by laboratory method 2. Failing that, an approximation may be made for moisture, eg. for cod 80%.

CALCULATIONS

A. Preparation of standard curve

1. From the volumes of standard BSA used calculate the total mg protein added to each test tube from the following:

   mg protein standard per tube

$$B = \frac{1000 \times W_1 \times V_2}{V_1} \qquad (1)$$

   Where:

   B = mg protein (assuming BSA to be 100% pure) in test tube
   W_1 = weight BSA standard in grams added to volumetric flask
   V_1 = volume of volumetric flask in ml
   V_2 = volume of aliquot of standard BSA added to test tube in ml

   N.B. 1 mg protein nitrogen equals 6.25 mg protein

2. Construct a standard curve with absorbance on the y-axis and mg protein added on the x-axis as shown in figure 17.5. Note the non-linearity beyond absorbance of 0.4.

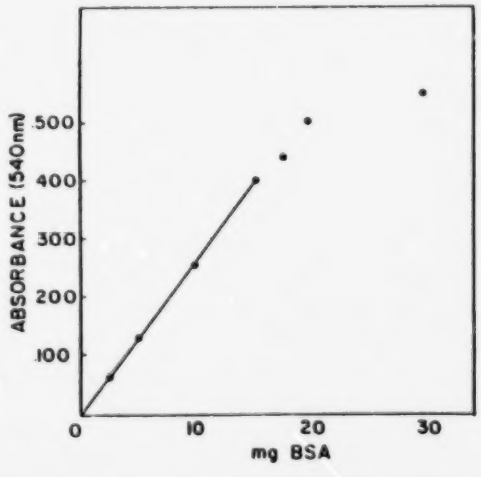


Figure 17.5 Typical standard curve for biuret analysis, absorbance vs mg BSA protein in tube.

B. Protein in extract

1. For each absorbance obtained subtract the absorbance of the corresponding turbidity blank.

2. From the graph for each net absorbance determine an equivalent protein content reading "R".

3. Salt extractable protein (SEP) content expressed as percent protein or g protein extracted per 100 g fish muscle can be determined by:

$$SEP = \frac{\text{protein per mg in tube} \times \text{total extracting volume in blender}}{\text{weight of fish extracted}} \times 100$$

$$= \frac{\frac{R}{V_3} \times V_4 + \frac{M \times W_2}{100}}{W_2 \times 1000} \times 100$$

$$= \frac{R \times [V_4 + (0.01 \times M \times W_2)]}{V_3 \times W_2 \times 10} \qquad (2)$$

Where:

M = moisture content (%) in fish, determined or approximated
R = reading of total mg protein in test tube obtained from graph (from net absorbance)
SEP = salt extractable protein expressed as percent protein extracted, i.e. grams protein extracted per 100 g fish
V_3 = aliquot (ml) extract added to test tube
V_4 = volume (ml) of buffer added to blender, usually 430 ml
W_2 = weight of fish used for extract, usually 22 g

N.B. Extractable protein nitrogen expressed as percent protein nitrogen extracted or grams nitrogen per 100 g fish may be obtained by dividing SEP by 6.25.

i.e.
$$EPN = \frac{SEP}{6.25} \qquad (3)$$

EXAMPLE

Frozen cod was subjected to EPN analysis by blending 22 g flesh with 430 ml buffer. For the analysis of protein 1.5 ml of extract produced an absorbance of 0.140 and a turbidity absorbance of 0.005. From the standard curve (Figure 17.5) a protein value of 5.1 mg was obtained. Moisture was estimated at 80%. From formula 2 salt extractable protein was determined to be:

$$SEP = \frac{5.1 \times [430 + (0.01 \times 80 \times 22)]}{1.5 \times 22 \times 10}$$

$$= \frac{5.1 \times 447.6}{1.5 \times 22 \times 10}$$

= 6.92 g protein / 100 g fish

Extractable protein nitrogen from formula 3 can be calculated as:

$$EPN = \frac{6.92}{6.25}$$

= 1.10 g protein nitrogen / 100 g fish

REFERENCES

1. BAILEY, K. 1944. Advances in Protein Chemistry 1:289-317.

2. REAY, G.A. 1933. J. Soc. Chem. Ind. 52:265.

3. LAUDER, J.T., and W.A. MacCallum. 1970. Keeping time of frozen redfish (Sebastes marinus mentelle) fillets in relation to handling of the raw material and storage temperatures after processing and freezing. J. Fish. Res. Bd. Canada 27:1589.

4. REAY, G.A. and C.C. Kuchel. 1937. Rep. Food Inv. Bd. G. Brit. 1936, 93-95, 1937.

5. DINGLE, J.R. 1967. Determination of salt-extractable protein in fresh and frozen fish. Personal comm.

6. DYER, W.J., H.V. French, and J.M. Snow. 1950. Extraction of protein fractions in fresh fish. J. Fish Res. Bd. Can. 7:585.

7. DYER, W.J., H. Brockerhoff, R.J. Hoyle, and D.I. Fraser. 1964. Polyphosphate treatment of frozen cod. I. Protein extractability and lipid hydrolysis. J. Fish. Res. Bd. Canada 21:101.

8. GILL, T.A., R. A. Keith, and B. Smith-Lall. 1979. Textural deterioration of red hake and haddock muscle in frozen storage as related to chemical parameters and changes in the myofibrillar proteins. J. Food Sci. 44:661.

10. DINGLE, J.R. and B. Lall. 1979. Stability of the minced flesh of argentine (Argentina silus) and roundnose genadier (Coryphaenoides rupestris) during storage at -10°C. Can. Inst. Fd. Sci. Technol. 12:40.

11. DINGLE, J.R., R.A. Keith, and B. Lall. 1977. Protein instability in frozen storage induced in minced muscle of flatfishes by mixture with muscle of red hake. Can. Inst. Fd. Sci. Technol. J. 10:143.

12. DYER, W.J. 1951. Protein denaturation in frozen and stored fish. J. Fish. Res. Bd. Canada 16:522.

13. CASTELL, C.H. 1971. Metal catalyzed lipid oxidation and changes of proteins in fish. J. Am. Oil Chem. Soc. 48:645.

14. CASTELL, C.H., and B. Smith. 1973. Measurement of formaldehyde in fish muscle using TCA extraction and the Nash reagent. J. Fish. Res. Bd. Canada 30:91.

15. CASTELL, C.H., B. Smith and W.J. Dyer. 1973. Effects of formaldehyde on salt extractable proteins of gadoid muscle. J. Fish. Res. Bd. Canada 30:1205.

16. CASTELL, C.H., B. Smith, and W. Neal. 1970. Effects of transition metal ions on the extractable protein of fish muscles. J. Fish. Res. Bd. Canada 27: 701-714.

17. NOWLAN, S.S., W.J. Dyer and R.A. Keith. 1975. Temperature and deteriorative changes in postrigor cod muscle stored up to 14 days in the superchill range -1 to -4°C. J. Fish. Res. Bd. Canada 32: 1595.

18. TOKUNAGA, T. 1974. The effect of decomposed products of trimethylamine oxide on quality of frozen Alaska pollock fillet. Bull. Jap. Soc. Sci. Fish. 40:167.

19. TOKUNAGA, T. 1964. Studies on the development of trimethylamine and formaldehyde in Alaska pollock muscle during frozen storage. I. Bull. Ho K Kaido Reg. Fish Res. Inst. 29:108.

20. DYER, W.J., and D.F. Hiltz. 1974. Sensitivity of hake muscle to frozen storage. Halifax Lab Fish. Mar. Serv. New Series Circular 45:4p.

21. KINGSLEY, G.R. 1939. J. Biol. Chem. 131:197.

22. SNOW, J.M. 1950. Proteins in Fish Muscle II Colorimetric estimation of fish muscle protein. J. Fish. Res. Bd. Canada 7:594.

23. UMIMOTO. 1966. Bull. Jap. Soc. Sci. Fish. 32:427.

24. Cowie, W.P. and I.M. Mackie. 1968. Examination of the protein extractability method for determining cold-storage protein denaturation in cod. J. Sci. Fd. Agric. 19:696.

18. DIMETHYLAMINE (DMA)

GENERAL DISCUSSION

Early investigations dealing with fish deterioration indicated that secondary amines were present in the muscles of several species (1). One of the amines, dimethylamine (DMA) was found to be produced simultaneously with formaldehyde (FA) (2, 3) under conditions of frozen storage (maximum formation ca -10°C). Accumulation of these products also resulted in a corresponding decrease in measurable trimethylamine oxide (4). An enzyme system, TMAO-ase, was thought to be responsible for the following reaction:

$$(CH_3)_3NO \xrightarrow{\text{enzyme}} (CH_3)_2NH + HCHO$$

Highest TMAOase activity occurs in marine fish species of the gadoid family and is proportional to the amount of dark lateral muscle present in the fillets; red hake has the most active system and haddock the lowest (5, 6, 7, 8). Highest enzyme activity was actually found in some of the visceral organs such as the liver and kidney (9, 2). Certain species of squid, bivalves and gastropods also produce DMA during frozen storage and processing (10, 11). Others such as flatfish (halibut, flounder, plaice, sole), ocean perch, rockfish, freshwater fish, scallops, lobsters, and shrimp are very low in TMAO or lack the TMAOase enzyme system (5, 12).

The localization of TMAOase activity has severe implications on frozen storage stability of deboned fish products where red and white tissues, sometimes from different species, are intimately mixed (13, 14, 15, 16, 17). Deterioration of minced meat occurs twice as fast as in intact fillets (17, 18). Consequences of formaldehyde and DMA production include loss of extractability of myofibrillar proteins (4), loss of water holding capacity, toughening and overall decrease in cooked acceptability (19, 15, 12, 17, 16, 20, 5).

Dimethylamine content in fish muscle was first determined as copper dimethyldithiocarbamate in 1938 by Reay using a procedure first developed by Dowden (21). The method was subsequently modified by Dyer (22) to ensure complete recovery of added dimethylamine from fish extracts and accepted as a measure of frozen fish quality in much the same manner as TMA was used for chilled fish quality (23). Freshly frozen fish have DMA values of 0.1 mg nitrogen per 100 g compared to 3 or 4 for fish stored frozen for a longer time. Originally, a trichloroacetic acid extract of the fish muscle was used but subsequent literature reports extraction with 6 percent perchloric acid (15, 8) since that extract may be used for other analyses.

Interference of DMA in the picric acid trimethylamine (TMA) test and the usefulness of measuring DMA and TMA together prompted development of a simultaneous DMA/TMA method by Castell and co-workers (23). Later investigations showed that TMA actually interferes in the dithiocarbamate DMA procedure resulting in lowered absorbance values (24) and thus imparting analytical error for frozen fish DMA after long periods in chilled or iced storage. The simultaneous TMA/DMA procedure was therefore recommended for the analysis (24) but with modified equations from those originally presented by Castell et al. (23). The new equations may have been a reflection of the sensitivity of the method to operator technique (23,24). The DMA/TMA procedure utilizes the discrepancy in DMA and TMA absorbance values when two different neutralizing agents, KOH and K_2CO_3, are used in the picric acid TMA test.

Recent investigators have turned to GLC (25) and HPLC (26) techniques for simultaneous TMA, DMA and NH_3 analyses.

a. Application

The dithiocarbamate DMA procedure is applicable to the measurement of dimethylamine in marine species which are prone to enzymatic TMAO breakdown in frozen storage (cusk, hake, pollock, cod). Since DMA and formaldehyde are formed in equal amounts, the DMA test is an indirect indicator of texture quality. When fish have been iced for a considerable length of time before freezing and have accumulated significant levels of

trimethylamine (TMA) from bacterial action, the simultaneous TMA/DMA procedure should be used to compensate for TMA interference in the dithiocarbamate method.

b. Principle

A trichloroacetic acid (TCA) extract of fish muscle is reacted with copper ammonium reagent and carbon disulphide at 40 to 50°C to produce a yellow coloured copper dimethyldithiocarbamate. The solution is acidified with acetic acid and shaken vigorously to facilitate extraction of the coloured complex into the benzene layer. After drying with sodium sulfate, absorbance of the solution is measured at 440 nm.

c. Precautions

1. TCA extracts are stable in frozen storage.
2. Carbon disulfide is foul smelling, extremely poisonous and explosive; exercise caution. The analysis should be performed in the fume hood as much as possible.
3. Tubes should be warm during acetic acid addition and shaking of step 8 for thorough benzene extraction. Vigorous shaking is important.
4. The presence of TMA from bacterial breakdown of TMAO lowers the DMA value obtained by this method. For samples containing TMA such as from fish stored chilled or on ice for several days, use the combined TMA/DMA analysis laboratory method 12.

SAMPLE PREPARATION

Defrost samples overnight under controlled conditions, +5°C for eg. Blend representative portion in food processor and weigh 50 g aliquots. Store refrigerated until extracted. Arrange defrost schedule to facilitate prompt sampling once material has thawed.

APPARATUS

1. **Vortex mixer**
2. **Water bath** set at 37-40°C
3. **Rotator** for mixing tubes
4. **Spectrophotometer** set at 440nm
5. **Blender** with small blender jar
6. **Low speed centrifuge** (optional)
7. **Glass wool**
8. **Filter paper** Whatman #4
9. **Glassware:** 20 x 150 mm screw capped test tubes, pasteur pipets, 100 ml volumetric flask

REAGENTS

1. **Stock DMA standard (0.2 mg DMA-N/ml):** In a 100 ml volumetric flask, dissolve 0.1165 g dried DMA-HCl in a small amount of distilled water. Make up to 100 ml with distilled water. Refrigerate.
2. **Working DMA standard (2 µg DMA-N/ml:** Dilute 1 ml DMA stock solution to 100 ml with distilled water. Refrigerate.
3. **Copper ammonium reagent**
   a. Dissolve 20 g ammonium acetate and 0.2 g $CuSO_4$ $5H_2O$ in 30 ml distilled water.
   b. Dissolve 10 g NaOH in 25 ml distilled water. Cool.
   c. Add the acetate solution to the NaOH solution.
   d. Add 20 ml concentrated NH_4OH.
   e. Make up to 100 ml with distilled water.
   f. Store in polyethylene bottle.
4. **Carbon disulphide (5%) in benzene:** Dilute 25 ml CS_2 to 500 ml with benzene.
5. **Acetic acid (30%):** Dissolve 150 ml glacial acetic acid in a portion of

distilled water. Make up to 500 ml with distilled water.

6. **Anhydrous sodium sulfate**, granular

7. **Trichloroacetic acid (7.5% w/w):** Dissolve 7.5 g TCA in 92.5 ml H_2O.

PROCEDURE

A. TCA extraction of samples

1. Add 50 g sample and 100 ml of 7.5% TCA (1:2 extraction) to a small blender jar and blend thoroughly.

2. Centrifuge at 4°C for 15 minutes at about 2000 x g (approximately 4000 rpm). An alternate purification procedure to centrifugation and decanting is filtration through Whatman #4 filter paper.

3. Decant supernatant fluid through glass wool.

4. Extracts may be frozen at -20°C for extended periods of time with little deterioration.

B. Analysis of TCA Extracts

1. Pipet aliquots (1-4 ml) of extract into 25 ml screw capped tubes (teflon lined).

2. For standard curve, pipet 1.0, 2.0, 3.0, 4.0, 5.0 and 7.0 ml of working DMA standard into tubes (to give final concentrations of 2, 4, 6, 8, 10 and 14 ug DMA).

3. Dilute both extracts and standards to 10 ml with distilled water. The blank consists of 10 ml distilled water.

4. Add 1 ml copper-ammonium reagent and mix on Vortex.

5. Add 12 ml 5% CS_2 in benzene. Place caps loosely on tubes.

6. Heat in water bath at 37 - 40°C for 5 minutes.

7. Tighten caps and mix on rotator for 5 minutes.

8. Immediately add 1 ml 30% acetic acid and agitate on Vortex for 1 minute. Do not allow tubes to cool before adding acetic acid. If they have, rewarm in water bath before addition of the acid. Uniformity of the shaking and mixing operation is critical. Be certain that layers do not separate during this stage.

9. Remove the top benzene layer into another tube and dry by adding a small amount of anhydrous $NaSO_4$.

10. Read absorbance of standards and samples at 440 nm.

CALCULATIONS

1. Plot absorbance of benzene solution vs. µg DMA nitrogen added to the test tube as shown in figure 18.1 below:

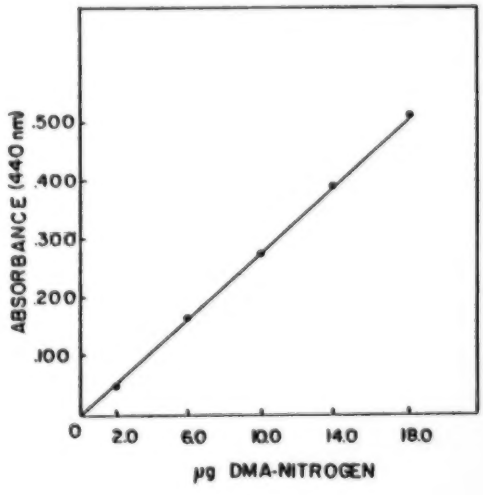


Figure 18.1 Typical standard curve for DMA; absorbance vs µg DMA-N added to test tube.

2. For each sample absorbance determine the equivalent µg DMA nitrogen from the graph.

3. Calculate DMA content from the formula:

$$\text{DMA} = \frac{\text{DMA in tube x total vol. extractant phase}}{\text{vol. added to test tube x wt fish extracted}}$$

$$= \frac{D \times [V_1 + (0.01 \times M \times W)]}{V_2 \times W \times 1000} \times 100 \qquad (1)$$

Where:

DMA = Dimethylamine expressed as mg DMA-N/100 g fish
D = equivalent DMA in µg determined from standard curve
M = moisture of fish sample expressed in percent
V_1 = volume in ml of TCA added for 1:2 extraction
V_2 = volume in ml of extract added to test tube
W = weight of fish used in 1:2 extraction

EXAMPLE

In a DMA analysis of cod flesh stored at -12°C for 8 months, a sample of 50 g (W) was extracted with 100 ml (V_1) TCA. A 1 ml (V_2) aliquot was used for the analysis. The final benzene solution had an absorbance of 0.225.

From the standard curve (Figure 18.1) the equivalent DMA in the test tube is determined to be 8.00 µg DMA nitrogen.

A moisture content of 80% will be assumed. DMA content of the fish from formula (1) may be calculated as:

$$\text{DMA} = \frac{8.00 \times [100 + (0.01 \times 80 \times 50)]}{1 \times 50 \times 1000} \times 100$$

$$= \frac{8.00 \times 140}{50 \times 1000} \times 100$$

= 2.24 mg DMA-N per 100 g fish

REFERENCES

1. BEATTY, S.A. 1938. Studies on fish spoilage. II. The origin of trimethylamine produced during the spoilage of cod muscle press juice. J. Fish. Res. Bd. Canada 4:63.

2. AMANO, K., and K. Yamada. 1965. The biological formation of formaldehyde in cod flesh. In FAO - The Technology of Fish Utilization (R. Kreuzer, Ed.) Fishing News (Books), London. pg. 73.

3. AMANO, K., and K. Yamada. Biological formation of formaldehyde in the muscle tissue of gadoid fish. Bull. Jap. Soc. Sci. Fish. 30:430.

4. TOKUNAGA, T. 1964. Series on the development of dimethylamine and formaldehyde in Alaska pollock muscle during frozen storage. Bull. Hokkaido Reg. Fish. Res. Lab. 29:108.

5. SHENOUDA, S.Y.K. 1980. Theories of protein denaturation during frozen storage of fish flesh. In Advances in Food Research, Academic Press, Inc. Vol. 26:275.

6. TOKUNAGA, T. 1970. Trimethylamine oxide and its decomposition in the bloody muscle of fish II. Formation of DMA and TMA during storage. Bulletin of the Japanese Society of Scientific Fisheries 36:510.

7. CASTELL, C.H., B. Smith, and W. Neal. 1971. Production of dimethylamine in muscle of several species of gadoid fish during frozen storage, especially in relation to presence of dark muscle. J. Fish. Res. Bd. Canada 28:1.

8. GILL, T.A., R. A. Keith, and B. Smith-Lall. 1979. Textural deterioration of red hake and haddock muscle in frozen storage as related to chemical parameters and changes in the myofibrillar proteins. J. Food Sci. 44:661.

9. DINGLE, J.R., J.A. Hines, and W. Robson. 1974. Frozen storage stability of minced fish. Environment Canada, Fisheries and Marine Service, New Series Circular No. 48.

10. RYU, B., J. Lee, and E. Lee. 1974. Changes of dimethylamine (DMA) content in fish muscle during heat processing. Bull Korean Fish. Soc. 7:115.

11. HARADA. 1975. Studies on enzymes forming formaldehyde and dimethylamine in fish and shellfish. J. Shimonosaki Univ. Fisheries 23:163.

12. CASTELL, C.H., W. Neal, and B. Smith. 1970. Formation of dimethylamine in stored frozen sea fish. J. Fish. Res. Bd. Canada 27:1685.

13. DINGLE, J.R. and B. Lall. 1979. Stability of the minced flesh of argentine (Argentina silus) and round-nose genadier (Coryphaenoides rupestris) during storage at -10°C. Can. Inst. Fd. Sci. Technol. 12:40.

14. DINGLE, J.R., R.A. Keith, and B. Lall. 1977. Protein instability in frozen storage induced in minced muscle of flatfishes by mixture with muscle of red hake. Can. Inst. Fd. Sci. Technol. J. 10:143.

15. DINGLE, J.R. and J.A. Hines. 1975. Protein instability in minced flesh from fillets and frames of several Atlantic fishes during storage at -5°C. J. Fish Res. Bd. Canada 32:775.

16. DYER, W.J., and D.F. Hiltz. 1974. Sensitivity of hake muscle to frozen storage. Environment Canada, Fisheries and Marine Service, New Series Circular No. 45.

17. HILTZ, D.F., W.J. Dyer, and D.W. Lemon. 1974. Some properties of fillets and minced flesh of silver hake (Merluccius hibinearis) in frozen storage. Environment Canada, Fisheries and Marine Service, New Series Circular No. 46.

18. BABBITT, J.K., D.L. Crawford, and D.K. Law. 1972. Decomposition of TMAO and changes in protein extractability during storage of minced and intact hake muscle. J. Agric. Food Chem. 20:1052.

19. CASTELL, C.H., B. Smith, and W. Neal. 1970. Effects of transition metal ions on the extractable protein of fish muscles. J. Fish. Res. Bd. Canada 27: 701-714.

20. SIKORSKI, Z., J. Olley, and S. Kostuch. 1967. Protein changes in frozen fish. In Critical Reviews in Food Science and Nutrition 8:97.

21. DOWDEN, H.C. 1938. The determination of small amounts of dimethylamine in biological fluids. Biochem. J. 32:455.

22. DYER, W.J., and Y. A. Mounsey. 1945. Amines in fish muscle II. Development of trimethylamine and other amines. J. Fish. Res. Bd. Canada 6:359.

23. CASTELL, C.H., B. Smith, and W.J. Dyer. 1974. Simultaneous measurements of trimethylamine and dimethylamine in fish, and their use for estimating quality of frozen stored gadoid fillets. J. Fish. Res. Bd. Canada 31:383.

24. MORAL, A., F. Jimenez-Colmenero, and A.J. Borderias. 1979. Contribution to the dimethylamine and trimethylamine measurement in frozen fish using Dyer's method. Proc. XV Int. Congress of Refrig. Vo. III:1053.

25. KUWATA, K., Y. Yamazaki, and M. Vebori. 1980. Determination of traces of low aliphatic amines by gas chromatography. Anal. Chem. 52:1980.

26. GILL, T.A., and J. W. Thompson. 1984. Rapid, automated analysis of amines in seafood by ion-moderated position HPLC. J. Food Science 49:603.

19. FORMALDEHYDE (FA)

GENERAL DISCUSSION

During frozen storage, especially at temperatures above -25°C, the osmoregulatory compound TMAO (1) present in marine species (gadoids in particular) enzymatically degrades to dimethylamine (DMA) and formaldehyde (FA) in equimolar amounts (2, 3, 4, 5). Formaldehyde is thought to be the agent predominantly responsible for the observed toughening, loss of protein extractability in salt solutions and general loss of organoleptic quality in frozen stored marine fish possessing the TMAO/TMAO-ase system (4, 6, 7, 8, 5, 9). However, there are other factors, yet unknown, involved in this deterioration process (5, 9, 10, 11, 12). Further information on this subject may be gained from the General Discussion sections of laboratory methods 18, 9, 12 and 17 for DMA, TMA, Simultaneous DMA/TMA and EPN.

The rate of formaldehyde accumulation is greater in minced fish than intact fillets, higher in dark muscle and viscera than white muscle, varies among species and appears not to be dependent upon TMAO concentration in the fish (12). However, fish stored for longer periods on ice before freezing formed less formaldehyde than fish frozen immediately after catching (6). Of the gadoids, the following species show an increased tendency to formaldehyde accumulation: haddock < cod < pollock < cusk < hake (7, 13). Mackerel, halibut, shark, sole, (6), plaice, halibut, redfish, and wolffish (13) do not form large amounts of formaldehyde in frozen storage.

Formaldehyde analytical procedures generally give very poor recoveries, in the range of 50% (8, 5, 14) due to the reactive nature of the compound. Formaldehyde is capable of interaction with several amines, amino acids, and protein functional groups (9). Since only "free" or "loosely bound" formaldehyde is measureable (15), Castell and Smith (8) suggested implementation of a "recovery factor" for the analysis. They also found that 10% trichloroacetic acid (TCA) or 10% perchloric acid were almost equally effective for the extraction step, giving recoveries of 52.7 and 49.2% respectively for cod flesh. Higher values were found for scallops and lobsters.

The formaldehyde analysis most commonly employed is that of Nash (16) in which formaldehyde is reacted with "Nash" reagent containing acetylacetone and excess ammonium salt to form diacetyldihydrolutidine. In newer applications however, the Nash reagent concentration has been doubled to increase shelf stability (15, 8).

a. **Application:**

The formaldehyde analysis is applicable to all fish species for the measurement of free (unbound) formaldehyde occurring naturally or produced by the TMA/TMAO-ase system in frozen storage. The method appears not to be affected by the presence of DMA or TMA but recoveries may differ between species (8).

b. **Principle:**

Free or "loosely bound" formaldehyde in a TCA or perchloric acid extract is reacted at 60°C with Nash color reagent containing acetylacetone with an excess of ammonium acetate to produce diacetyldihydrolutidine, measurable spectrophotometrically at 415 nm. For total formaldehyde a series of recovery tests may be undertaken as described by Castell (8).

c. **Precautions:**

1. Caution must be exercised when working with perchloric acid. According to the Merck Index, perchloric acid combines vigorously with water with evolution of heat, is extremely caustic and can burst into flame when in contact with oxidizable matter (paper, wood, etc.)

2. The Nash reagent should be stored in a dark place at refrigerator temperatures. Double strength reagent was found to be stable for a period of six months if properly cared for.

3. Absorbance values should not exceed 0.7

since the standard curve is non-linear beyond this value.

4. Tubes should not be heated longer than the five minutes prescribed at 60°C. Extra incubation will result in lowered absorbance values.

SAMPLE PREPARATION

Defrost samples overnight under controlled conditions, +5°C for eg. Blend representative portion in food processor and weigh 100 g aliquots. Store refrigerated until extracted. Arrange defrost schedule to facilitate prompt sampling once material has thawed.

APPARATUS

1. **Blender**
2. **Filter paper** Whatman #1
3. **pH meter** set at pH 7
4. **Vortex mixer**
5. **Water bath** set at 60°C
6. **Spectrophotometer** set at 415 nm
7. **Glassware:** 125 ml Erlenmeyer flask, 500 ml beaker, 18 x 150 mm culture tubes, 1 liter volumetric flask, 10 ml burette

REAGENTS

1. **Perchloric acid (6%):** In a 1 liter volumetric flask, weigh 85.7 g of 70% reagent grade $HClO_4$. Dissolve in a small amount of distilled water and dilute to volume.
2. **Potassium hydroxide (30%):** Dissolve 30 g KOH in 65 ml distilled water. Cool and dilute to 100 ml.
3. **Standard buffer solutions** commercially available pH 6 and 8
4. **Nash reagent:**
   a. Combine 2 ml acetyl acetone with 75 - 80 ml distilled water in a 125 ml Erlenmeyer flask. Cap and shake vigorously.
   b. In a 500 ml beaker, dissolve 150 g ammonium acetate in 300 ml water.
   c. Combine the above solutions and dilute to 500 ml.
   d. Make fresh daily and store refrigerated.
5. **Formaldehyde** (1 M aqueous) In a 100 ml volumetric flask, weigh 8.12 g of 37% w/w formaldehyde solution and dilute to volume with distilled water.

PROCEDURE

A. Perchloric acid extraction of samples

1. Blend a preweighed portion (100g) of homogenized fish in a blender for 2 minutes with twice the volume of 6% $HClO_4$ (200 ml).
2. Let the mixture settle for a few minutes.
   Caution: Perchloric acid is explosive and may ignite when spilled on cellulose materials (eg. wood, paper, etc.)
3. Filter the extracts through fluted Whatman #1 filter paper. Collect 50 ml aliquots. These aliquots may be frozen at -30°C for later analysis.

b. Neutralization of extracts

1. Carefully standardize the pH meter at pH 6 and 8, using commercial standard buffer solutions.
2. Neutralize 50 ml aliquots of the perchloric acid filtrates to pH 7 by careful dropwise addition of 30% KOH from a 10 ml graduated pipet or buret. Caution should be exercised in adjusting the pH - do not exceed pH 7. Record the volume of KOH used.

3. The neutralized extracts are not stable for long periods of time, so prompt analysis is essential. Acid extracts (not neutralized) may be frozen at -30°C until analysis.

C. **Determination of formaldehyde**

1. Pipet an aliquot (1 to 5 ml) of perchloric acid extract into an 18 x 150 mm test tube.

2. Standard curve:

   a. Prepare a set of standards by diluting 1 ml of 1 M HCHO to 100 ml with distilled water.

   b. Pipet 2 mls of this solution and dilute to 100 ml with distilled water.

   c. Withdraw 0, 1, 2, 3, and 4 ml aliquots to give final HCHO concentration of 0, 0.2, 0.4, 0.6 and 0.8 μ moles.

3. Dilute all samples and standards to 5 ml with distilled water.

4. Add 5 ml Nash reagent and mix well on Vortex.

5. Heat tubes for 10 minutes in a 60°C water bath.

6. Cool in cold water for 5 minutes.

7. Read absorbance at 415 nm.

CALCULATIONS

1. Prepare a standard curve as illustrated in Figure 19.1.

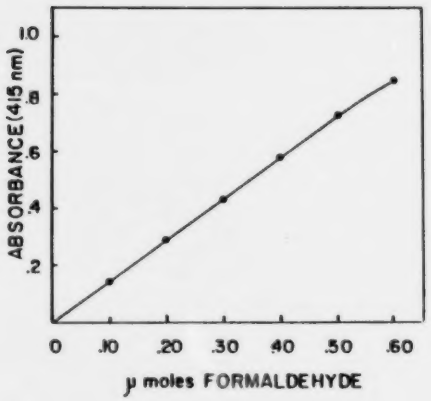


Figure 19.1 Typical formaldehyde standard curve; absorbance vs μ moles formaldehyde added to tube.

2. Formaldehyde concentration, expressed in μ moles / g fish:

$$FA = \frac{F \times (M + V_1)}{V_3 \times W} \times \frac{(50 + V_2)}{50} \qquad (1)$$

Where:

F = μ moles formaldehyde read from standard curve
M = moisture content of fish, expressed in percent
V_1 = volume (ml) of perchloric acid added for 1:2 extractions
V_2 = vol (ml) of KOH used to neutralize sample
V_3 = vol (ml) of extract added to tube
W = weight of fish used in 1:2 extraction

3. Formaldehyde concentration, expressed in μg/g fish:

$$FA = \frac{F \times (M + V_1)}{V_3 \times W} \times \frac{(50 + V_2)}{50} \times G \qquad (2)$$

Where G = 30 i.e. gram molecular weight of formaldehyde

EXAMPLE

A 100 g (W) sample of previously frozen haddock was analyzed for formaldehyde content. Exactly 1.2 ml (V_2) of KOH was used to neutralize a 50 ml aliquot of a perchloric acid extract of the fish. Three ml (V_3) of neutralized extract was added to the test tube for analysis and gave a formaldehyde concentration of .079 μ moles (F) from the standard curve. Given a moisture content (M) of 81.2%, a formaldehyde concentration may be calculated from formula 1:

$$FA = \frac{.079 \times (81.2 \times 200)}{3 \times 100} \times \frac{(50 + 1.2)}{50}$$

$$= 4.30 \times 1.024$$

$$= 4.4 \text{ μ moles/g fish}$$

From formula 2,

$$FA = \frac{.079 \times (81.2 \times 200)_x (50 + 1.2)_x 30}{3 \times 100 \qquad 50}$$

$$= 4.30 \times 1.024 \times 30$$

$$= 132.1 \text{ μg/g fish}$$

REFERENCES

1. LOVE, R.M. 1970. The Chemical biology of fishes. Academic Press, New York.

2. SHENOUDA, S.Y.K. 1980. Theories of protein denaturation during frozen storage of fish flesh. In Advances in Food Research, Academic Press, Inc. Vol. 26:275.

3. AMANO, K., and K. Yamada. 1965. The biological formation of formaldehyde in cod flesh. In FAO - The Technology of Fish Utilization (R. Kreuzer, Ed.) Fishing News (Books), London. pg. 73.

4. DINGLE, J.R. and J.A. Hines. 1975. Protein instability in minced flesh from fillets and frames of several Atlantic fishes during storage at -5°C. J. Fish Res. Bd. Canada 32:775.

5. CASTELL, C.H., B. Smith and W.J. Dyer. 1973. Effects of formaldehyde on salt extractable proteins of gadoid muscle. J. Fish. Res. Bd. Canada 30:1205.

6. TOKUNAGA, T. 1964. Studies on the development of dimethylamine and formaldehyde in Alaska pollock muscle during frozen storage. Bull. Hokkaido Reg. Fish. Res. Lab. 29:108.

7. CASTELL, C.H. 1971. Metal catalyzed lipid oxidation and changes of proteins in fish. J. Am. Oil Chem. Soc. 48:645.

8. CASTELL, C.H., and B. Smith. 1973. Measurement of formaldehyde in fish muscle using TCA extraction and the Nash reagent. J. Fish. Res. Bd. Canada 30:91.

9. CONNELL, J.J. 1975. The role of formaldehyde as a protein crosslinking agent acting during the frozen storage of cod. J. Sci. Fd. Agric. 26:1925.

10. CASTELL, C.H., B. Smith, and W. Neal. 1970. Effects of transition metal ions on the extractable protein of fish muscles. J. Fish. Res. Bd. Canada 27: 701-714.

11. GILL, T.A., R. A. Keith, and B. Smith-Lall. 1979. Textural deterioration of red hake and haddock muscle in frozen storage as related to chemical parameters and changes in the myofibrillar proteins. J. Food Sci. 44:661.

12. SIKORSKI, Z., J. Olley, and S. Kostuch. 1967. Protein changes in frozen fish. In Critical Reviews in Food Science and Nutrition 8:97.

13. CASTELL, C.H., B. Smith, and W. Neal. 1971. Production of dimethylamine in muscle of several species of gadoid fish during frozen storage, especially in relation to presence of dark muscle. J. Fish. Res. Bd. Canada 28:1.

14. KWON, T. and B.M. Watts. 1964. Malonaldehyde in aqueous solution and its role as a measure of lipid oxidation in foods. J. Food Sci. 29:294.

15. COCHIN, J., and J. Axelrod. 1959. Biochemical and pharmacological changes in the rat following chronic administration of morphine, nallorphine, and normorphine. J. Pharmacol. Exp. Ther. 25:105.

16. NASH, T. 1953. The colorimetric estimation of formaldehyde by means of the antzsch reaction. Biochem. J. 55:416.

20. G/P VALUES

GENERAL DISCUSSION

Many of the objective chemical tests for measuring fish freshness such as trimethylamine (TMA), dimethylamine (DMA) and total volatile bases (TVB) essentially measure bacterial spoilage (1). However as fish spoils, it passes through the following sequence of events:

rigor mortis - dissolution of rigor mortis - autolysis

Therefore, freshness of fish must also be related to biochemical changes as fish will spoil under aseptic conditions through natural enzyme degradation (2). In particular, autolytic and biochemical deteriorations in fresh fish become more important when proper chilling and handling are used.

Post-mortem nucleotide degradation in most fish muscle proceeds primarily via the following sequence of reactions (3):

ATP → AMP → IMP → INO → Hx → X → U

where ATP is adenosine-5'-triphosphate, ADP is adenosine-5'-diphosphate, AMP is adenosine-5'-monophosphate, IMP is inosine-5'-monophosphate, INO is inosine, X is xanthine, and U is uric acid. There are various stages in this degradative sequence which could be considered as indices of quality such as dephosphorylation of IMP or the formation of Hx.

ATP is rapidly degraded to AMP and subsequently to IMP during or shortly after the death struggle by partial dephosphorylation and deamination (4, 1). The dephosphorylation of IMP is primarily autolytic (5) and occurs during the period of early chilled storage. The disappearance of IMP has been correlated with a loss of fresh fish flavour in some species (6, 7, 8). Hx accumulation in fish tissue reflects the initial phases of autolytic deterioration as well as later contributions through bacterial spoilage (9, 10, 6, 2). In addition, nucleotides also appear to be fairly stable during heat processing (12) and irradiation (7).

While useful indices of freshness have been based on individual nucleotide or breakdown products accumulation (13, 14, 7, 10, 4, 5, 15) indicators that incorporate the measurement of several of these nucleotides are advantageous. Multiple measurements remove some sample to sample and species to species variations with only a slight loss in sensitivity. Ehira (2) has described a K value based on the concentrations of various nucleotides and their breakdown products estimated from the following formula:

$$K = \frac{INO + Hx}{ATP + ADP + AMP + IMP + INO + Hx} \times 100 \quad (1)$$

while Karube et al (16) described a K_1 value as:

$$K_1 = \frac{INO + Hx}{IMP + INO + Hx} \times 100 \quad (2)$$

Both of these values are based mainly on the appearance or disappearance of IMP and describe a period of early chill storage not measured by such objective chemical tests as the TMA test. In fact by the time substantial amounts of TMA accumulate, fish are in incipient stages of spoilage (5, 17). The most serious limitation of the above indicators is that the reaction is completed well within the edible storage life of a number of fish species (10, 11).

Burns et al (18) have proposed a G value based on the accumulation of Hx but also reflecting the disappearance of IMP, AMP and INO. The index is useful over the entire iced shelf-life of the lean species of fish studied:

$$G = \frac{Hx + INO}{INO + IMP + AMP} \quad (3)$$

A second quality indicator, the P value (18), serves as an indicator of spoilage during the early stages of chill storage:

$$P = \frac{Hx + INO}{INO + IMP + Hx + AMP} \quad (4)$$

G and P values can provide a basis for the establishment of a grading system. G and P values have been established for several fish species (18) by comparisons with other fish freshness tests and physical evaluations.

a. **Application**

G and P values are applicable to most fish samples although species variations can be expected. Both are sensitive to deterioration in the early stages of refrigerated storage while G values are also applicable to the later stages of deterioration. These values may also be applied to frozen stored samples. P and G values are most useful with lean fish as other factors such since rancidity in fatty fish may render the product undesirable before significant G and P values are obtained.

b. **Principle**

The analysis (18) utilizes a high performance liquid chromotography (HPLC) method for determining ATP degradation product in biological samples. Nucleotides are extracted with 0.6 M perchloric acid and determined by HPLC using a reversed phase column and UV absorbance detection (254 nm). The mobile phase is 0.01 M phosphate buffer (pH 4.5) at 0.5 ml/min. G and P values are obtained through a calculation involving the concentrations of the appropriate nucleotides or degradation products.

c. **Precautions**

1. Fresh sample flesh should be kept chilled, excised and blended quickly with the extracting acid medium, while frozen samples should be processed without thawing to retard enzymatic activity in the flesh and prevent further sample deterioration. IMP and AMP especially are temperature sensitive.

2. Caution must be exercised when working with perchloric acid. According to the Merck Index, perchloric acid combines vigorously with water, with evolution of heat, is extremely caustic and can burst into flame when in contact with oxidizable matter (paper, wood, etc.)

3. Extracts may be stored frozen after neutralization without undue loss of nucleotides.

4. Fresh mobile phase should be prepared daily.

SAMPLE PREPARATION

Comminute a representative sample of fish flesh in a food processor taking care to maintain the sample chilled. Preweigh 5 g aliquots and freeze or extract as soon as possible. Neutralized extracts may be stored without change when frozen.

APPARATUS

1. **Food processor.**

2. **Filter paper** Whatman #1.

3. **Blender.**

4. **LC pump** - operated at 0.5 - 1.0 ml/min.

5. **LC injector** - loop injector with 20 µl loop.

6. **Detector** - UV operated at 254 nm.

7. **Recorder** - strip chart.

8. **LC column** - RP-2, RP-8 or RP-18 HPLC reversed phase analytical column 4.6 mm id x 10 cm, 10 um particle size fitted with an RP-2, RP-8 or RP-18 respectively, HPLC guard column 4.6 mm id x 3 cm, 10 um particle size (Brownler Labs Inc. Santa Clara, CA). Most reverse phase columns are acceptable.

9. **Aqueous sample clarification kit** - PN 26865, used to remove fine particles of 0.45 m or greater (Waters Associates, Inc., Milford, MA).

10. **Glassware** - 10, 50, 500 and 1000 ml volumetric flasks, 200 ml blender flasks.

REAGENTS

1. **Perchloric acid** (0.6 M): with caution slowly add 32.3 ml concentrated perchloric acid (60%) to a 500 ml volumetric flask containing approximately 400 ml distilled water. Make up to volume with distilled water.

2. **Potassium hydroxide/phosphate buffer (pH 7.6):** dissolve 8.16 g KH_2PO_4 in approxi-

mately 60 ml distilled water and adjust to pH 7.6 with 50% KOH. Dilute to 100 ml with distilled water.

3. **50% KOH:** dissolve 50 g KOH in 50 ml distilled water and cool to room temperature.

4. **LC mobile phase:** 0.01 M potassium phosphate buffer pH 4.5. Dissolve 1.36 g KH_2PO_4 in approximately 400 ml double-distilled water, adjust to pH 4.5 with KOH or H_3PO_4 as necessary and dilute to 1 litre. Methanol should be used as a modifier if required.

5. **Standards:** (a) stock solutions. Prepare individual stock standards for LC by dissolving 0.010 g Hx, INO, IMP and AMP respectively in 40 mls distilled water (agitation such as mixing with a spin bar may be required, especially with Hx). Dilute to the mark in 50 ml volumetric flasks. (Dilute standards 1:10 before injection on the LC.)

   (b) mixed standards (working solutions). Pipet 0.125, 0.250, 0.375 and 0.50 ml of each stock solution of IMP and Hx into four separate 10 ml volumetric flasks. Add 0.25, 0.50, 0.75 and 1.0 ml respectively of each stock solution of AMP and INO to the flasks. Dilute to the mark with distilled water. These four solutions contain 2.5, 5.0, 7.5 and 10 µg/ml solutions of IMP and Hx and 5.0, 10, 15 and 20 µg/ml of AMP and INO. Mixed standards solutions are stable for approximately 2 weeks when stored at 0-4°C.

PROCEDURE

a. Preparation of standard curve

1. Set flow rate of mobile phase at 0.5 - 1.0 ml/min and let column and detector equilibrate 20 - 25 minutes.

2. Inject 10 µl aliquots of each mixed standard solution.

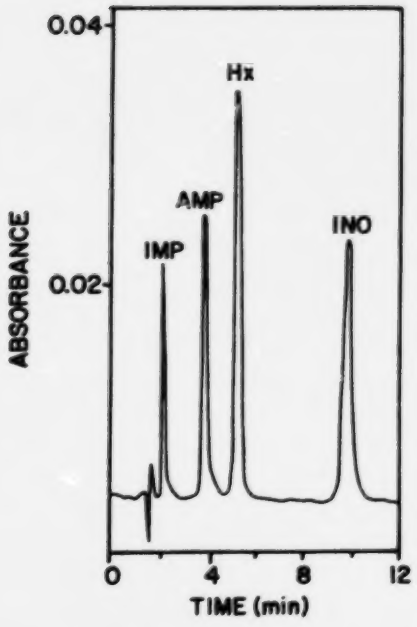


Figure 1. Typical standard curves for nucleotide determinations at 254 nm using Hx, IMP, AMP and INO as standards.

3. Determine absorbances of the various nucleotides from peak heights recorded at 254 nm.

4. Plot peak height vs µg injected to provide standard curves (Figure 1).

5. Prepare standard curves at least twice per day to assure accurate quantitation.

b. Sample Preparation

1. Without thawing, blend a preweighed 5 g portion of finely chopped fish flesh at maximum speed for 2 minutes with 50 ml of 0.6 M perchloric acid.

2. Suction filter contents through Whatman #1 paper using a small amount of distilled water to rinse the blender flask.

3. Mix the filtrate well and record the volume (V_1) in a graduate cylinder.

4. Transfer a 1.0 ml aliquot of the filtrate to a screw top test tube containing 1.0 ml KOH phosphate buffer (pH 7.6). Mix the solution, cool to 0-4°C, decant and filter through the aqueous clarification kit.

5. Inject aliquots (V_2) of neutralized filtrate directly for LC analysis.

6. Dilute with distilled water if necessary (D).

CALCULATIONS

a. Concentrations of individual nucleotides and degradation products.

Hx content in fish tissue is calculated from the following equation:

$$\text{Hx content } (\mu \text{ moles/g}) = K\ S\ V_1\ D\ /\ H\ V_2\ W \qquad (5)$$

where:

- S = peak height (mm)
- H = slope of standard curve (mm/ µg)
- V_1 = total volume of perchloric extract plus wash (ml)
- D = dilution factor of neutralized extract before LC
- V_2 = injection volume for LC (µl)
- W = weight of sample (g)
- K = 14.71 (µl)(µ moles)/(mg)(µl) a constant which takes into account the 1:1 dilution during neutralization.

IMP, AMP and INO are concentrations are calculated from equation 5 substituting the following values for K: 5.75, 5.76 and 7.46 (µl)(µ moles) / (µg)(ml) respectively.

b. G and P values

G and P values are based on the accumulation and/or degradation of Hx, IMP, AMP and INO and are calculated from equations 3 and 4 where Hx, IMP, AMP and INO represent the concentrations (µ moles/g) of the Hx, IMP, AMP and INO respectively (Figures 3 and 4).

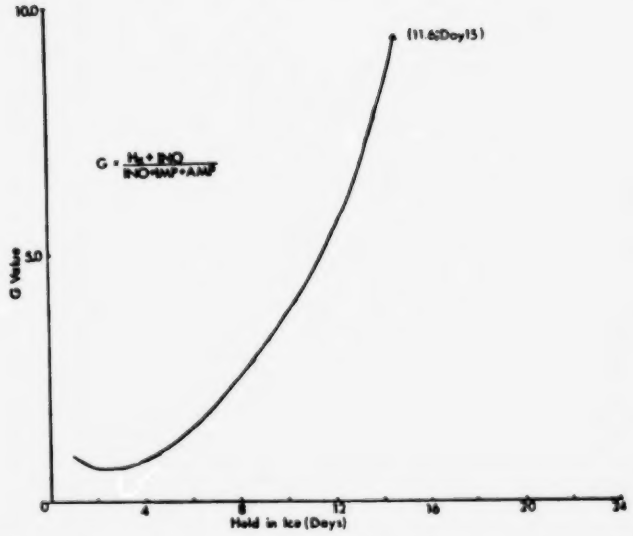


Figure 2. Typical G value changes for commercially caught cod bled and gutted at sea and subsequently stored on ice.

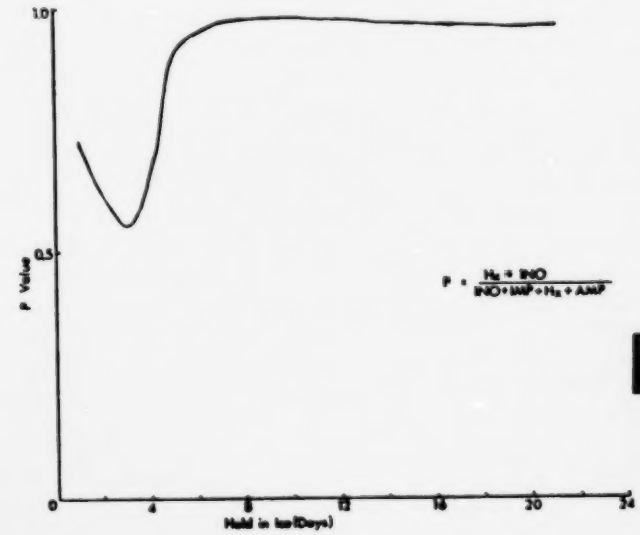


Figure 3. Typical P value changes for commercially caught cod bled and gutted at sea and subsequently stored on ice.

EXAMPLE

A 5.7 gram (W) sample of aquarium held cod was blended with 50 ml 0.6 M perchloric acid. The total volume of the extract plus wash was 66 ml (V_1). Slopes of the standard curves (H) were 3125, 1338, 1838 and 1312 mm/ ug for Hx, INO, IMP and AMP respectively. A 10 µl (V_2) injection of the neutralized clarified filtrate was injected directly onto the LC after a 1:10 dilution (D = 10). Peak heights (S) of 23, 29, 104 and 12 mm resulted for Hx, INO, IMP and AMP respectively.

$$\text{Hx content (}\mu\text{ moles/g)} = \frac{1.47 \times 23 \times 66 \times 10}{3125 \times 10 \times 5.7}$$

$$= 1.25$$

$$\text{INO content (}\mu\text{ moles/g)} = \frac{7.46 \times 29 \times 66 \times 10}{1338 \times 10 \times 5.7}$$

$$= 1.87$$

$$\text{IMP content (}\mu\text{ moles/g)} = \frac{5.75 \times 104 \times 66 \times 10}{1838 \times 10 \times 5.7}$$

$$= 3.76$$

$$\text{AMP content (}\mu\text{ moles/g)} = \frac{5.76 \times 12 \times 66 \times 10}{1312 \times 10 \times 5.7}$$

$$= 0.610$$

Therefore, from equation (3), the G value is:

$$G = \frac{1.25 + 1.87}{1.87 + 3.76 + 0.610} = 0.50$$

While from equation (4), the P value is:

$$P = \frac{1.25 + 1.87}{1.87 + 3.76 + 1.25 + 0.610} = 0.42$$

REFERENCES

1. MARTIN, R.E., R.J.H. Gray, M.D. Pierson, 1978. Quality assessment of fresh fish and the role of naturally occurring microflora. Food Technol. May:188.

2. EHIRA, S., 1976. A biochemical study on the freshness of fish. Bull. Tokai Reg. Fish. Res. Lab. No. 88.

3. SAITO, T., A. Arai, and M. Matsuyashi, 1959. A new method for estimating the freshness of fish. Bull. Jap. Soc. Sci. Fish. 24:749.

4. JONES, N.R. and J. Murray, 1964. Rapid measures of nucleotide dephosphorylation in iced fish muscle. Their value as indicies of freshness and of inosine-5'-monophosphate concentration. J. Sci. Food Agric. 15:684.

5. JONES, N.R., 1965. Hypoxanthine and other purine containing fractions of fish muscle as indicies of freshness. In "The Technology of Fish Utlization", ed. R. Kreuzer. Fishing News (Books) Ltd., London. p. 179.

6. FRASER, D.I., D.P. Pitts and W.J. Dyer, 1968. Nucleotide degradation and organoleptic quality in fresh and thawed mackerel muscle held at and above ice temperature. J. Fish. Res. Bd. Can. 25:239.

7. SPINELLI, J., G. Pelroy and D. Miyouchi, 1969. Quality indicies that can be used to assess irradiated seafoods. In "Freezing and Irradiation of Fish", ed. R. Kreuzer. Fishing News (Books) Ltd., London, U.K. p. 425.

8. JONES, N.R. and J. Murray, 1961. Nucleotide concentration in codling (*Gadus callarias*) muscle passing through rigor mortis at 0°C. Z. Vergleich Physiol 44:174.

9. JONES, N.R. and J. Murray, 1962. Degradation of adenine and hypoxanthine - nucleotide in the muscle of chill-stored trawled cod (Gadus callarias). J. Sci. Food Agric., 13: 475.

10. JONES, N.R., J. Murray, E.I. Livingston and C.K. Murray, 1964. Rapid estimations of hypoxanthine concentrations as indicies of the freshness of chill-stored fish. J. Sci. Food Agric. 15:763.

11. KASSEMSARN, B., B. Sanz Perez, J. Murray and N.R. Jones, 1973. Nucleotide degradation in the muscle of iced haddock (Gadus aeglefinus), lemon sole (Pleuronecetes microcephalus) and plaice (Pleuronecetes platessu). J. Food Sci. 28:28.

12. HUGHES, R.B. and N.R. Jones, 1966. Measurement of hypoxanthine concentration in canned herring as an index of the freshness of the raw material with a comment on flavour relations. J. Sci. Fd. Agric. 17:434.

13. BURNS, B.G. and P.J. Ke, 1985. Liquid chromotographic determination of hypoxanthine in fish tissue. J. Assoc. Off. Anal. Chem. 68:444.

14. BURT, J.R., 1977. Hypoxanthine: a biochemical index of fish quality. Process. Biochem. 12:32.

15. BEUCHAT, L.R., 1973. Hypoxanthine measurement in assessing freshness of chilled channel catfish (Sctalurus punctatus). J. Agr. Food Chem. 21:453.

16. KARUBE, I., H. Matsuoka, S. Suzuki, E. Watanabe, and K. Toyama, 1984. Determination of fish freshness with an enzyme sensor. J. Agric. Food Chem. 83:314.

17. SPINELLI, J., M. Eklund, and D. Miyouchi, 1964. Measurement of hypoxanthine in fish as a method of assessing freshness. J. Food Sci. 29:710.

18. BURNS, B.G., P.J. Ke and B.B. Irvine, 1985. Objective procedure for fish freshness evaluation based on nucleotide changes using a HPLC system. Can. Tech. Rep. Fish. Aquat. Sci. No. 1373, 35 p.

E. PHYSICAL ATTRIBUTES

21. COLOR MEASUREMENT

GENERAL DISCUSSION

When light strikes an object some of that light is reflected from the surface, some is scattered, some is absorbed and some passes through (if the object is translucent). Light which is not absorbed gives the object its visual character (gloss, color). The human observer can differentiate among about 10 million juxtaposed surface colors but can identify only about 300 from memory (1). Perceived color character is also subject to object surface character, background, viewing angle, and angle of illumination as well as spectral quality, intensity and angular size of the light source used for illumination. Therefore when it is necessary to describe color of an object for identification or quality purposes, viewing conditions must be strictly controlled or alternately (and preferably) an objective (instrumental) means of color discrimination and measurement should be employed. To become more familiar with color the reader should consult one of the several detailed references available on the subject (1, 2, 3). The following information is condensed from the book by R.S. Hunter (1).

When the search for a mathematical description of color was initiated, the object was to select three standard primaries that would facilitate the identification of color stimuli by numbers. In 1931 a set of functions $\bar{x},\bar{y},\bar{z}$ representing three imaginary red, green, and blue lights were adopted which for each wavelength were called the CIE tristimulus values for that wavelength. However since the observed color of an object is not a single wavelength but rather a range of wavelengths (spectral curve), in order to represent that color in the $\bar{x},\bar{y},\bar{z}$ system, individual $\bar{x},\bar{y}$ and $\bar{z}$ values must be recorded together with corresponding intensity factors for each wavelength over the visible range. If S_λ is an intensity factor then for a specific color, all $\bar{x}$ contributions made by each wavelength must be combined by addition as shown below in order to represent all of the light at all wavelengths for each primary:

$$\text{combined } \bar{x} = X$$
$$= S_{\lambda 1}\bar{x}_{\lambda 1} + S_{\lambda 2}\bar{x}_{\lambda 2} + S_{\lambda 3}\bar{x}_{\lambda 3} + \ldots + S_{\lambda n}\bar{x}_{\lambda n}$$
$$= \int_{400}^{700} S_\lambda \bar{x}_\lambda \, d\lambda$$

Three new tristimulus values X,Y and Z were established to represent the above integrals of $\bar{x},\bar{y}$ and $\bar{z}$.

However the attributes X,Y and Z are not very useful for assessment of visual color attributes since while Y correlates with lightness, X and Z do not correlate with hue, saturation or any visually meaningful attribute of color appearance. Three new values x,y and z called chromaticity co-ordinates or trichromatic coefficients were proposed. These were defined as:

$$x = \frac{X}{X+Y+Z} \qquad y = \frac{Y}{X+Y+Z} \qquad z = \frac{Z}{X+Y+Z}$$

Since $x+y+z = 1$, only two were required to specify chromaticity and from these meaningful relationships correlating with color were developed. Color was defined in terms of Y, x, and y even though values of x,y and z do not individually correlate with perceived color.

Tristimulus values are measured with a constant illuminant since chromaticites of colored objects "move" or "shift" to some degree with change of illuminant in the same direction as do x and y of the illuminant. Chromaticities of neutral colored objects are more susceptible to this shift than strongly colored objects. It should be noted that the human eye does not fully perceive this shift in chromaticity with illuminant change due to the "color constancy" phenomenon. Differences in light level and spectral distribution are compensated by the human observer; ie. an apple appears red under sunlight, tungsten light, or fluorescent light. Photographs taken under the three illuminant conditions differ markedly and to some degree reflect the chromaticity shifts of the objects. A neutral subject photographed with "daylight" balanced film will appear normal under daylight, yellowed under tungsten and

yellow-green under fluorescent illumination. Therefore the requirement for standardized viewing conditions for human observer judgements is imperative.

Several solutions to the chromaticity shift problem of the x,y system were proposed. One of these was the introduction of X%,Y% and Z% which expressed tristimulus values as percent of X,Y and Z of the standard white surface. However the CIE X,Z, x and y color scales were found to be inadequate for representing the color of objects since they were not originally designed for that purpose. They were difficult to relate to perceived colors and the spacing of colors in the system was not uniform as related to visual differences. Thus several alternate systems were developed, each with specific notation and application, but mathematically interconvertible (as an approximation) and having in common, dimensions related to the MacAdam/Scofield/Hunter lightness "L", redness-greenness "a", and yellowness-blueness "b" values.

Modern color measurement instruments are either spectrophotometer or tristimulus instruments. The former provide wavelength-by-wavelength analyses of light reflecting or transmitting properties while the latter use filters to approximate spectral functions and elucidate color in terms of X,Y,Z or L, a,b (and occasionally R_d, a,b). While these instruments are less flexible than the eye they provide under the correct measuring conditions values which correlate well with visual evaluations.

Many transformations have evolved for inter-sample comparison of color data. The L,a,b diagram in Figure 20.1 demonstrates the three dimensional nature of the more popular Hunter L,a,b color system or color solid. In the system a plus value of "a" indicates redness and a minus value greenness while a plus value of "b" indicates yellowness and a negative, blueness. "L" denotes lightness. Sometimes the color difference between two objects is determined by the value "E" below:

$$E = \sqrt{(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2}$$

An example of application of the L,a,b system has been with tomatoes where if the a/b ratio was between 0.2 and 2.0 this value became a good index of hue (4). Cot^{-1} a/b, i.e. arc cot a/b (the angle which has tangent equal to b/a), has also been applied to the definition of this parameter. Color intensity was also approximated by $(a^2+b^2)^{0.5}$ successfully for spinach puree (5). Literature dealing with fishery products has generally been limited to the reporting of L,a,b values (6, 3, 7); color grade of canned sockeye salmon was predicted with 91% accuracy using the a/b ratio of the raw material (3).

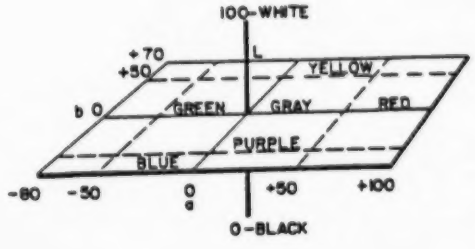


Figure 20.1 The L,a,b system depicted graphically.

a. Application

Color measurement is applicable to all materials which may be presented to the color difference meter. Different materials require individual preparation and presentation methods as described in the "Sample Preparation Section".

b. Principle

The color difference meter compares an unknown specimen with standards of predetermined color characteristics (8). Since instruments are prone to source/filter/photocell combination error it is critical that the instrument be calibrated immediately before use to a color standard as close as possible to the color of the sample. After the standardization step the sample is placed on the circular specimen "port", the color of the "surface" is measured on the flat plane, and X,Y,Z or L,a,b values are recorded from the instrument.

c. **Precautions**

1. The instrument should be allowed to warm-up for at least 1/2 hour prior to use.

2. The instrument must be standardized accurately (and patiently) with a color standard (available from the Gardner or Hunter Companies) very closely approximating that of the sample.

3. Sample surface must be flat and presented in a standard manner - see Sample Preparation Section.

4. If sample is cold, frosting of the observation port may occur.

5. Careful attention must be paid to sample representativeness, homogeneity, orientation, and opacity.

6. If samples are air sensitive ie. discolor on standing, this factor should be overcome in the procedure.

7. Unsteady readings in the instrument may be due to dirty contacts in the controls. Dials and switches should be activated several times prior to standardization or measurement.

SAMPLE PREPARATION

Samples must be representative and presented in the same manner each time measured. If published methods exist for presentation, these should be followed. The following are guidelines for sample preparation and presentation:

1. Opaque homogeneous sample

   Measured surface must be flat. If the material is homogeneous and opaque, the sample may be cut to provide a flat representative (reproducible) surface. If sample is pliable it should be pressed down and held to promote a flat measurable surface.

2. Wet sample

   The instrument must be protected from water. Sample if wet should be placed on a clear glass or plastic plate. If the sample is cold, plate may fog on instrument side.

3. Liquid sample

   Petri plates may be used for sample presentation of liquids. The presence of particulate matter can influence readings. If the liquid is not opaque a black box should be used to cover the plate and port. Use the same amount of liquid each time.

4. Curved objects

   The small sample port should be installed for measuring color of curved objects. The black box should cover specimen to prevent outside light from influencing readings.

5. Translucent materials

   Use a large window size when measuring color of translucent objects. The black box should cover the sample and port.

PROCEDURE

The following procedure is for color measurement using the Gardner Color Difference Meter. Instruction manuals should be consulted for the particular instrument used. However, the principle of measurement will be the same for all instruments.

1. Allow instrument to warm up for at least 1/2 hour before beginning standardization.

2. Choose a color standard plate (supplied with instrument or obtained from the Gardner or Hunter companies) as close as possible to the color of the sample.

3. Choose the maximum size sample port suitable for the sample. Place standard color tile on the port ensuring complete coverage. If the sample is to be measured through a glass or plastic plate, standardization should also occur through this material.

4. With instrument in "read" position press "Y", "X" and "Z" settings sequentially and repeatedly until stable readings are obtained.

5. Adjust readings to correspond to readings printed on the back of the color tile beginning with "Y". Adjust "X" and "Z" similarly. An adjustment of "Y" will affect "X" and "Z" calibration. If the instrument was recently calibrated and readings have shifted, likely re-calibration of "Y" will cause "X" and "Z" readings to come back into calibration without individual adjustment.

   Unsteady readings may be due to dirty contacts. Activate dials and switches several times to facilitate better contact.

6. Place sample over the port and cover with black box to shield from outside light. Sample must be flat - see Sample Preparation Section.

7. Press in order "Y", "X" and "Z" switches and record values.

8. Repeat with new sample or with different orientation. Several readings should be taken.

CALCULATION

When X,Y and Z are expressed as decimal fractions, L,a and b values may be calculated from the following equations:

$$Rd = Y \times 100 \qquad (1)$$

$$L = 10 \times (Rd)^{0.5} \qquad (2)$$

$$f_y = 0.51 \frac{(21 + 20Y)}{(1 + 20Y)} \qquad (3)$$

$$a = 175\, f_y\, (1.020\, X - Y) \qquad (4)$$

$$b = 70\, f_y\, (Y - 0.847Z) \qquad (5)$$

EXAMPLE

Redfish skin color was measured on the Gardner Color Difference meter using a glass plate over the sample port. Three readings were taken on each side of the fish representing head, middle and tail areas. One of the readings obtained from the centre section was:

Y=27.6%, X=30.4%, and Z=20.7% or Y=0.276, X=0.304, and Z=0.207.

Using equations 1 to 5, L, a,b values are calculated as:

$Rd = 100 \times 0.276 = 27.6$

$L = 10 \times (27.6)^{0.5} = 52.5$

From equation 3:

$$f_y = 0.51 \frac{(21 + [20 \times 0.276])}{(1 + [20 \times 0.276])}$$

$f_y = 2.074$

From equation 4:

$a = 175 \times 2.074\ ([1.020 \times 0.304] - 0.276)$
$= 12.37$

$b = 70 \times 2.074\ (0.276 - [0.847 \times 0.207])$
$= 14.62$

Therefore, L,a,b values for the redfish are 52.5, 12.37 and 14.62 respectively.

REFERENCES

1. HUNTER, R.S. 1975. The Measurement of Appearance. John Wiley & Sons, Toronto.

2. MACKINNEY, G. and A.C. Little. 1962. Color of Foods. AVI Publishing Co., Inc., Westport, Conn.

3. FRANCIS, F.J. and F.M. Clydesdale. 1975. Food Colorimetry, Theory and Applications. AVI Publishing Co., Inc., Westport, Conn.

4. FRANCIS, F.J. 1975. The origin of $\tan^{-1} a/b$. J. Food Sci. 40:412.

5. CLYDESDALE, F.M. and F.J. Francis. 1969. Colorimetry of foods. I. Correlation of raw, transformed and reduced data with visual rankings for spinach puree. J. Food Sci. 34:349.

6. LINDSAY, R.C., D.A. Stuiber, B. Stewart and V.C. Carlson. 1981. Evaluation of Burbot (Lota lota) acceptability for processing. Can. Inst. Food Sci. Technol. J. 14:196.

7. MOTOHIRO, T. and Y. Sato. 1981. Food quality of cured pink salmon packed in different kinds of packaging film during cold storage. Bull. Fac. Fish. Hokkaido Univ. 32:194.

8. ANONYMOUS. Technical Information on Gardner Color Difference Meters. Gardner Laboratory Inc., Bethesda, Maryland. Bull. pH 260-72.

APPENDIX A

DEVELOPMENT OF FISH GRADING STANDARDS BY USING SCIENTIFIC QUALITY ASSESSMENT PROCEDURES.
P.J. Ke, 1983. Presented at the First National Fisheries Technology Conference, Washington, D.C., November 1983.

SCIENTIFIC CHARACTERISTICS OF FISH

In the laboratory, fish may be considered a biological-chemical entity. The meat is composed of about 75-80% water, 16-20% protein, 1-10% fat, and traces of inorganic salts and minerals. The first component, water, is the life-giving substance surrounding and suspending other molecules and acting as the medium in which biochemical reactions take place. Removal of this phase, as in the drying process, preserves the product by effectively stopping or greatly retarding deleterious reactions. The fat content of fish is relatively low and although fat oxidizes during storage to produce off-flavours, the overall effect of lipid deterioration on fish quality may be considered minimal and becomes only important during frozen storage. The remaining component, therefore, which is responsible for the decrease of quality is protein.

Protein is an ordered array of very large molecules and predominantly of structural function. However, one protein class, the enzymes, is very active in life-sustaining biochemical reactions. The delicate state of enzyme equilibrium and control maintained by body regulators fails when an organism dies and from that point enzymatic reactions proceed unhindered. Since the circulatory system has stopped, raw materials for building are quickly depleted and only degradative reactions continue; structural proteins are reduced to smaller subunits by their "molecular cousins", the enzymes. Not only are these by-products foul smelling and putrid to the taste, but in fish some may produce undesirable texture denaturation and discolorations. In some fish, particularly herring and mackerel, digestive enzymes of the viscera can literally digest their way through the belly flap. This extreme case of enzyme action is termed "belly burst".

Naturally occurring enzymes are not the only source of degradative activity in stored fish. While living, all organisms have protective mechanisms barring the entrance of bacteria. When life processes stop, bacteria quickly invade the body and secrete powerful enzymes to begin the digestion of tissues. Therefore, at death, fish enter a state of uncontrolled enzymatic protein degradation from both natural and bacterial sources which results in the accumulation of peptides, amines, ammonia and other nitrogen containing compounds. While this chaotic state is undesirable for preservation, a study of the levels of these by-products makes it possible to develop methods for the assessment of fish quality.

Although the fat content of groundfish is low and is not likely to influence the short-term storage quality, fish liver is very high in fat, approximately 20%. Since fats are susceptible to enzymatic and chemical oxidation reactions, the fatty fish is greatly altered during iced and frozen storage. This factor may not specifically affect eating quality but is a useful phenomenon from a quality monitoring standpoint, in particular to rancidity development.

SCIENTIFIC QUALITY ASSESSMENT

Various quality parameters which are sensitive, reliable, reproducible and change as a function of quality deterioration and keeping time, can be selected and applied to fish quality evaluation. Comprehensive reviews on quality indicators have been initiated in our laboratory. The most useful quality parameters are total volative bases, total volatile acids, extractable proteins, hypoxanthine value, trimethylamine value, dimethylamine value, thiobarbituric acid-reactive substances value, free fatty acid value, peroxide value, carbonyl value, pH value, dielectric behaviour, polyene index and texture index, etc.

In order to develop standards for selling-point grading, the following rules must be considered when selecting quality parameters for the laboratory evaluation:

(1) to select at least two indicators for your operation;
(2) two of the three types of quality changes; micro-organism, enzymatic and chemical reactions must be covered;
(3) the reacting substances should be tested in a matrix such as proteins, fats, water, indication compounds, etc.

The relative variations for laboratory evaluations of fish have been summarized in Tables 1 and 2. With good control, the overall errors can be maintained at no more than 10%.

RELATIVE ERRORS IN LABORATORY QUALITY EVALUATION	%
Operational	0-10
Correlational	5-20
Seasonal	10-25
Methodology	5-40
Sampling	5-60

Table 1. Relative errors in laboratory quality evaluation.

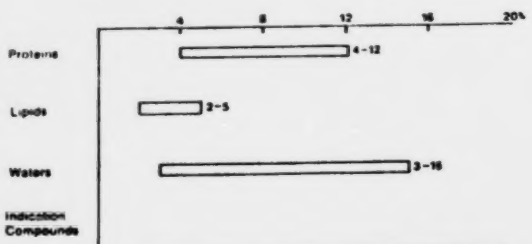


Table 2. Overall errors in various laboratory evaluations.

The procedure for selecting quality parameters for laboratory quality assessment of fish is described using squid as an example. Quality changes of squid samples held at +2°C in terms of TVB, TMA, and FFA values have been summarized in Figures 1, 2 and 3 respectively. The broken lines indicate suggested limits for Grade A (excellent) and Grade B (acceptable) quality and have been established from sensory data for both TVB and TMA values.

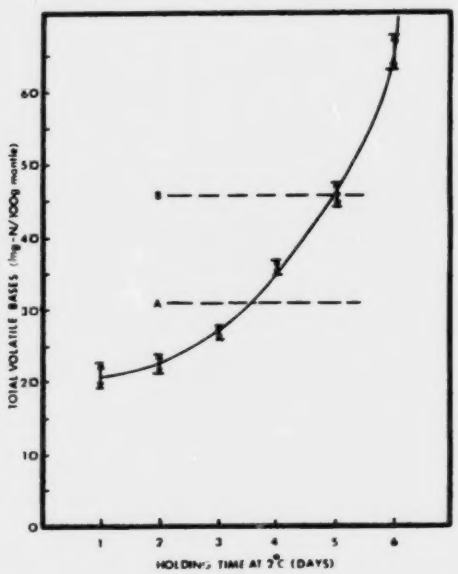


Figure 1. Changes in total volatile bases (TVB) over time for squid held in air at +2°C.

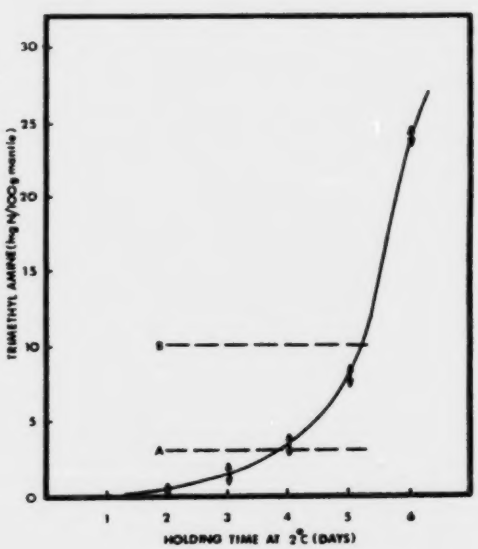


Figure 2. Changes to trimethyl amine (TMA) over time for squid held in air at +2°C.

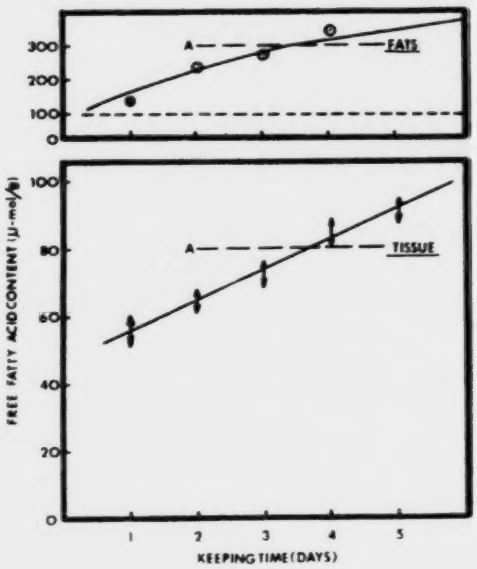


Figure 3. Changes in free fatty acid content (FFA) in liver tissue and liver fats over time for squid kept in air at +2°C.

Our preliminary data indicates a potential third indicator of fresh squid quality based on the accumulation of products of enzymatic fat hydrolysis, i.e. the measurement of free fatty acids (FFA). Squid contains a high portion of triglycerides and unsaturates. The increase of FFA in both liver tissue and liver oil as a function of holding time is depicted in Figure 3. However, the small overall change and larger deviation may limit the usefulness of this analysis. A more extensive study must be completed before FFA data can be applied objectively to the assessment of overall squid quality.

STANDARD DEVELOPMENT FOR SELLING POINT GRADING

The detailed operation of quality standard development has been illustrated in Figure 4. As in previous reports, fish grades have been divided into two/three classes; Grade A of excellent quality and Grade B/C of acceptable quality. The A, B/C division is the point at which the organoleptic panel first is able to detect the quality change in the project at $P \leq 0.05$ by non-parameric statistical treatment. Last class or Grade F is of unacceptable quality and is rejected by the panel at $P > 0.05$ by parametric

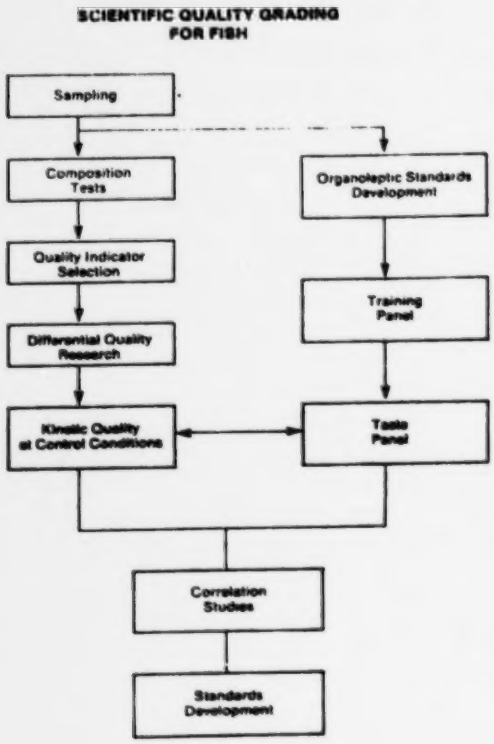


Figure 4. Scientific quality grading for fish.

analysis. An example of the grading standards for squid and groundfish is presented in Appendices A and B. The comparative evaluation of quality assessment by scientific evaluation and selling point operation is compared in Figure 5.

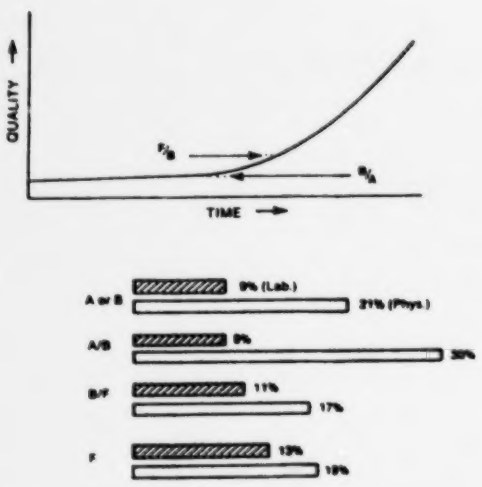


Figure 5. Variations on fish grading by physical and laboratory methods.

In addition to the grading operation, as shown in Figure 5, the overall quality assessment determined by laboratory tests and physical grading (selling point or dockside evaluation) has been summarized in Figures 6 and 7 from the results of the past three years. It can be concluded that physical grading can be employed satisfactorily for fresh groundfish and shellfish with less than 15% error on an accept/reject grade and 25% on quality differential evaluation. Scientific (laboratory) tests have demonstrated much better sensitivity and reproducibility (Table 3), but these methods are still limited when applied to field operations. However, scientific quality evaluation using selected parameters should provide a useful tool for developing quality standards of fish for various field grading operations.

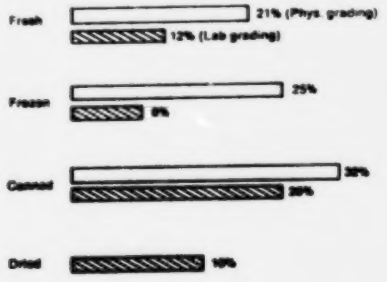


Figure 6. Comparison of overall errors between physical and chemical quality grading.

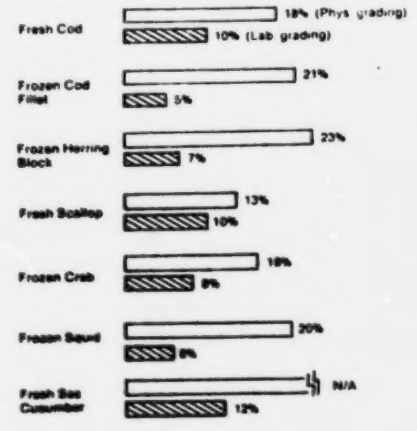


Figure 7. Comparison of overall errors for some selected samples.

SOME CONSIDERATIONS ON SCIENTIFIC FISH QUALITY ASSESSMENT
Advantages:
more specific
more reliable and reproducible
relatively simpler and faster
good for diff. evaluation
good for R/D projects
Disadvantages:
limited application to field
requires some apparatus and lab
slightly higher costs
scientific staff

Table 3. Some considerations on scientific fish quality assessment.

REFERENCES

Cox, H.E. and D. Pearson, 1962. "Chemical Analysis of Foods". Chem. Pub. Co. Ltd., New York, pp. 321.

Dyer, W.J., 1945. Amines in fish muscle, I. Colorimetric determination of trimethylamine as the picrate salt. J. Fish. Res. Bd. Can. 6:351.

Iwamoto, M. and H. Uchiyama, 1969. Effect of chemical ice on quality keeping of marine products. Bull. Tokai Reg. Fish. Res. Lab. 60:185.

Jangaard, P.M. and R.G. Ackman, 1965. Lipids and component fatty acids of the Newfoundland squid, Illex. J. Fish. Res. Bd. Can. 22:131.

Joseph, J., P.R. Girija Varma and R. Venkataraman, 1977. Iced and frozen storage of squid. Fish. Technology, 14:13.

Ke, P.J. and A.D. Woyewoda, 1978. A titrimetric method for determination of free fatty acids in tissues and lipid with ternary solvents and m-cresol purple indicator. Anal. Chim. Acta 99:387.

Ke, P.J., B. Smith-Lall and A.B. Dewar, 1981. Quality improvement investigation for Atlantic queen crab. Can. Tech. Rep. Fish. Aquatic Sci. No. 1002.

Ke, P.J., B. Smith-Lall and A.W. Woyewoda, 1981. Methodological investigation of fish quality evaluation in terms of TBA and FFA procedures. Presented in 25th AFTC, Norfolk, VA. August 16-19, 1981.

Larmond, E., 1977. "Laboratory methods for sensory evaluation of food". Agriculture Canada, Pub. No. 1637.

Matsumoto, J.J., 1958. Some aspects on the water soluble proteins of squid mantle. Bull. Tokai Reg. Fish. Res. Lab. 20:65.

Robles, C., E. Cervantes and P.J. Ke, 1982. Recommended method for testing the objective rancidity development in fish based on TBARS formation. Can. Tech. Rep. Fish. Aquatic Sci. No. 1089.

Siegel, S., 1956. "Non-parametric statistics for behavioural sciences". McGraw-Hill Co., Toronto, pp. 166.

Takagi, M., A. Lida and S. Oka, 1971. On the formation of unvolatile amines during putrefiction in the muscle of squid and octopus. Bull. Jap. Soc. Sci. Fish. 37:1079.

Takahashi, T., 1956. Biochemical studies on viscera of cuttlefish (ommastrephes sloani pacificus). II on the nitrogenous compounds hot water extract of liver. Bull. Jap. Soc. Fish. 43:1233.

Woyewoda, A.D. and P.J. Ke, 1980. Laboratory Quality Assessment of Canadian Atlantic Squid. Can. Tech. Rep. Fish. Aquatic Sci. No. 902.

APPENDIX - B

I. GUIDE FOR GRADING OF GROUNDFISH

(COD, POLLOCK, REDFISH, and FLOUNDER)

- INTRODUCTION -

This operational procedure guide was prepared as an aid to personnel involved in dockside, plant, and laboratory grading of groundfish. The guide does not introduce any new standards but provides a detailed step-by-step outline of the procedure to be followed when grading groundfish, using the Department of Fisheries and Oceans Proposed Grading Plan for Groundfish (page 139). It describes the assignment of Grades A, B, C, and reject to the fish, and supplies a sample score sheet (pages 130 and 131) which should be employed for recording scores and calculating the final grade for a lot of fish.

Table 1 HANDLING PRACTISES AND TEXTURE

In Table 1, scores are assigned to the criteria as Grade A, B, C, or reject. The grade for the whole section is the **lowest** grade assigned to any criterion within that section. Record scores for each fish graded on score sheet on page 131.

Handling practices are the first indicator of the quality of fish being landed. Notes should be made regarding:

1. general condition of fishing vessel;
2. condition of hold, fish boxes, or containers; and
3. quality and distribution of ice.

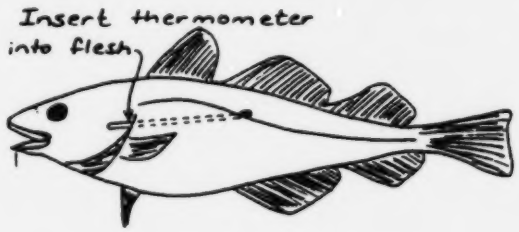
PROCEDURE	GRADES
1. **Select the fish to be sampled** To determine the number of fish required: a. Randomly select 10 fish from the lot. b. Determine the average weight per fish as: $\frac{\text{(total weight)}}{10}$ c. Number of fish in lot = $\frac{\text{Actual wt of whole lot}}{\text{Average wt per fish}}$ d. Based on the number of fish in the lot, use Table on page 132, to determine the number of fish to be sampled and proceed.	
2. **Observe handling practises** For cod and pollock, check each fish for proper gutting and bleeding. Open the abdominal cavity and inspect for removal of viscera and internal organs. Any gut still adhering to the abdominal cavity should be less than 1% of the total weight. The belly of the fish should not be split past the anus. Select grade.	Grade A - fish properly gutted, bled, and washed Grade B - fish not properly gutted, bled, and washed
Flounder are sometimes not gutted at sea, but should be bled by either throat slashing or bobtailing. Select grade.	Grade A - fish properly bled and washed Grade B - fish not properly bled and washed
Redfish are rarely bled or gutted at sea, so are not downgraded for failure to do so.	Grade A

3. Record temperature

For all species, in order to assess whether fish has been properly iced at sea, determine its temperature. Insert a thermometer into the collar of the fish and push it through the flesh to a point midway down the flank.

Ensure that the tip of the thermometer is completely embedded in the flesh. Leave it in place for about 1 minute before reading and recording temperature.

Any accurate thermometer that can be inserted into the flesh is suitable. A dial or probe type may provide the least resistance. Accuracy of thermometer should be checked; temperature of ice and freshwater mixture is 0°C.

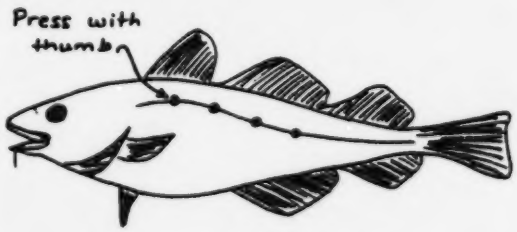


Grade A - temperature of 4°C (40°F) or less

Grade B - temperature greater than 4°C (40°)

4. Assess texture of fish flesh

For all species, press thumb along lateral line for the anterior two-thirds of the fish. Do not press along the tail section, as it contains little flesh and mostly bones, and will not give a true indication of texture.



Grade A - flesh is firm and resilient, and springs back immediately when released.

Grade B - reasonably firm, some loss of resiliency, thumb indentations slowly fill out.

Grade C - moderately soft, thumb indentations may remain in flesh.

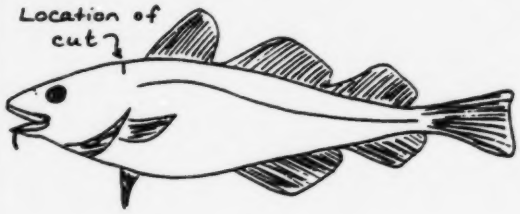
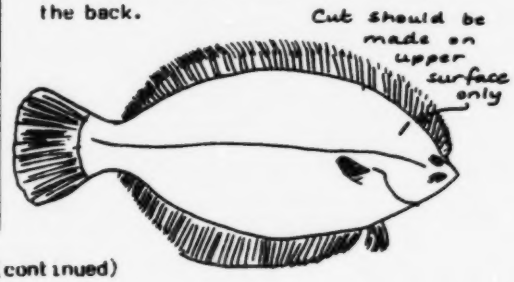
Reject - excessively soft flesh.

Table 2 EXAMINATION IN ROUND, GUTTED, OR HEADED FORM

After examining the texture and handling practises (Table 1), complete Table 2 for each round, gutted, or headed fish.

In Table 2, the scores are assigned based on a defect point system. Each criterion may be given defect points ranging from 0 to 3. After completion of the whole section, these points are totalled and divided by five in order to arrive at the final grade for Table 2 as follows:

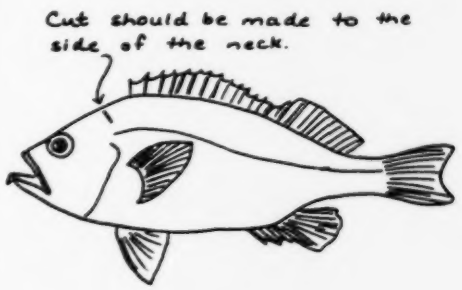
Grade A	less than 2 defect points
Grade B	2.0 to 2.4 defect points
Grade C	2.5 to 2.8 defect points
Reject	more than 2.9 defect points

PROCEDURE	DEFECT POINTS
1. Assess odour at neck	
For cod and pollock, using a sharp knife, make a 1 to 2 cm deep cut across the back of the neck just behind the gills. Spread the cut apart and determine odour by placing exposed flesh within 1 cm of nose. Do not cut more than 2 cm into the neck, because gill odours may be detected through the flesh.	0 - characteristic odour, fresh 2 - neutral, total absence of odour; characteristic odour no longer detectable but off-odours haven't developed Reject - off-odour, sour, putrid, bilgy, ammonia, unnatural odour.
	
For flounder, the cut should be made on the upper surface only, not through to the back.	0 - characteristic odour, fresh 2 - neutral, total absence of odour; characteristic odour no longer detectable but off-odour haven't developed Reject - off-odour, sour, putrid, bilgy, ammonia, unnatural odour.
	

(continued)

1 continued

For redfish, the cut should be made to the side of the neck, to avoid the spiny fin on the back.



0 - characteristic odour, fresh

2 - neutral, total absence of odour; characteristic odour no longer detectable but off-odours haven't developed

Reject - off-odour, sour, putrid, bilgy, ammonia, unnatural odour

2. **Assess odour of gills**

For all species, grasp the bony coverings of the gills and pull them apart to expose and separate the gills. Examine the odour by placing the gills within 1 cm of the nose.

0 - characteristic of species, fresh

1 - neutral - total absence of odour, characteristic odor no longer detectable but off-odours haven't developed

2 - faint sour odour

3 - slight to moderate sour odour

Reject - very sour, strong, or putrid

3. **Examine general appearance of fish**

For all species, look at both sides of the fish and examine its overall condition, giving particular attention to the skin.

0 - good overall appearance; skin lustrous and shiny, no fading

1 - good overall appearance, very slight bleaching of skin

2 - some loss of metallic lustre, some bleaching

3 - bloom gone from skin, color faded and bleached

4. Examine eyes

For all species, closely examine the eyes on both sides of the head. It is essential to consider both eyes, so that a damaged eye (punctured by ice or fork; frozen; or flattened by other fish) is not mistaken for an eye which is sunken or cloudy from poor handling or aging. Assign the grade for the best eye.

0 - clear, bright, convex eyes

1 - slightly sunken or somewhat dull

2 - dull and/or cloudy

3 - very dull, sunken, and cloudy

5. Note the appearance of the gills

For all species, pull the bony gill coverings apart and examine the gills closely for color and presence or absence of mucus.

0 - bright red, little mucus

1 - red, some mucus

2 - pinkish red to brownish, some mucus

3 - brown, may be covered with mucus

Table 3 EXAMINATION OF CUT SURFACES

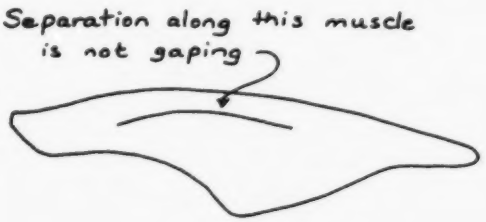
After completing the examination of the round, gutted, or headed fish, one fillet is removed per fish, and used for examination of the cut surfaces as required by Table 3. When filleting redfish, a diagonal cut should be made from just above the gills to behind the gut cavity, to avoid cutting into the viscera.

In Table 3, the scores are assigned as Grades A, B, C, or reject. The final grade for the section is the lowest letter grade assigned to any criterion in that section.

PROCEDURE	GRADE
1. Examine fillets for blood clots	
For all species, closely check both sides of the fillets for blood clots. Clots are free blood on the surface or open to the surface (eg. blood around a fork hole) which are larger than a 1/2 cm diameter circle (as designated by the grading card developed by DFO). Measure blood clots on the square centimeter grid provided on the DFO grading card.	Grade A - no blood clots greater than the 1/2 cm circle Grade B - no combination of blood clots exceeding 4 square cm total area (4, 1x1 squares on DFO measuring template) in any one fillet Grade C - one or any combination of blood clots which exceed 4 square cm total area in any one

2. **Assess texture of fillets**

For all species, the texture grade is a combination of the firmness component of the fish along with the amount of gaping and raggedness in the fillets. Using the cut side of the fillets, press the flesh with thumb or forefinger to determine resiliency. For determination of raggedness and gaping, use the picture guide on pages 23 and 24 of the DFO Grading Standard for Fresh and Frozen Atlantic Groundfish Products. Do not downgrade the fillets for a separation of the major muscle along the back; this is a result of manipulation during filleting, and is not indicative of poor quality.



Grade A - uniform, firm fillets with little or slight gaping.

Grade B - reasonably firm, resilient flesh with moderate gaping.

Grade C - moderately soft flesh with excessive gaping. Ragged or torn fillets permitted.

Reject - excessively soft flesh with unacceptable amount of gaping

3. **Determine the amount of chalkiness and/or jelliness**

For flounder, chalkiness and/or jelliness are sometimes encountered. Chalkiness is a condition of the flounder involving opaque, white, and dried flesh (appearance like freezer-burn). In jelliness, the flesh appears wet, shimmering, and jellylike, and is gelatinous to the touch.

For cod, pollock, and redfish, chalkiness and jelliness are not seen.

Grade A - No jelly. None or slightly chalky

Grade B - Slightly jellied or moderately chalky

Grade C - Moderately jellied or heavy chalky

Reject - Heavy jellied. Do not reject based on chalkiness

4. Examine the fillet for discoloration

Remove the skin and look at the both sides of the fillets for any abnormally discolored areas, i.e. do not consider very slight green or yellow hues as discoloration. Measure discoloration using the square centimeter grid on the DFO grading card (template).

For cod and pollock, pink or red discoloration is occasionally seen as a result of bruising.

For flounder, check for yellow or green (or pink) discoloration.

Grade A - no single discoloration, nor any combination, exceeding 2 square cm (i.e. two, 1x1 cm squares on grid of DFO measuring template) in any one fillet

Grade B - no single discoloration, nor any combination, exceeding 5 square cm in any one fillet

Grade C - any single discoloration, or combination the total surface area of which does not exceed 50% of the total surface area of any one fillet

Reject - any single discoloration or combination which exceeds 50% of the total surface of any one fillet

5. Assess odour of fillets

For all species, using the skin side of the fillets, determine odour by placing the nose within 1 cm of the fillet surface.

Grade A - odour characteristic of species

Grade B - neutral, total absence of odour

Grade C - slight off-odour, but not objectionable

Reject - any objectionable odour

CALCULATION OF THE FINAL GRADE

Tabulate the data on score sheet (page 131). For Table 2 add awarded defect points divide by 5 to determine the average score, and assign the grade for that section. The final overall grade of each fish is the lowest letter grade assigned to that fish in Table 1, 2, or 3. From final grades for all fish examined in the lot calculate percent (%) in each grade category. Grade for the lot is assigned by examining the lowest category first (reject) and working progressively upwards. The first grade in which 10% or more of the fish are classed becomes the grade of the lot. If jelliness or chalkiness are present at high levels, the cut-off point becomes 15%. An entire lot of fish shall be rejected if more than 10% of the sampled fish are rejected. When a lot is rejected, the owner may cull the fish and request a re-grading, the results of which are final.

Grading Guide for Iced Groundfish - key

ROUND FISH	Grade A	Grade B	Grade C	Reject
handling practise	fish properly gutted and washed	fish not properly gutted and washed		
temperature	temperature of 4°C (40°F) or less	temperature greater than 4°C (40°F)		
texture of fish	flesh is firm and resiliant, and springs back immediately when released	reasonably firm, some loss of resiliancy, thumb indentations slowly fill out	moderately soft thumb indentations may remain in flesh	excessively soft flesh

ROUND FISH	0	1	2	3	R
neck odour	characteristic odour, fresh		neutral, total absence odour; characteristic odour no longer detectable but off-odours haven't developed		off-odour, sour, putric bilgy, ammonia, unnatural odour
gill odour	characteristic of species, fresh	neutral, total absence of odour, characteristic odour no longer detectable but off-odours haven't developed	faint sour odour	slight to moderate sour odour	very sour, strong or putrid
general appearance	good overall appearance skin lustrous and shiny no fading	good overall appearance very slight bleaching of skin	some loss of metallic lustre, some bleaching	bloom gone from skin, color faded and bleached	
eyes	clear, bright, convex eyes	slightly sunken, or somewhat dull	dull and/or cloudy	very dull, sunken, and cloudy	
gill appearance	bright red, little mucus	red, some mucus	pinkish red to brownish some mucus	brown, may be covered with mucus	

FILLETS	Grade A	Grade B	Grade C	Reject
blood clots	no blood clots greater than 1/2 cm circle	no combination of blood clots exceeding 4 sq cm total area (4, 1x1 squares on DFO measuring template) in any one fillet	one or any combinations of blood clots which exceed 4 square cm total area in any one fillet	
texture of fillets	uniform, firm fillets with little or slight gaping	reasonably firm, resiliant flesh with moderate gaping	moderately soft flesh with excessive gaping. Ragged or torn fillets permitted	excessively soft flesh with unacceptable amount of gaping
chalkiness and/or jelliness	no jelly. none or slightly chalky	slightly jellied or moderately chalky	moderately jellied or heavy chalky	heavy jellied. Do not reject based on chalkiness
discoloration	no single discoloration, nor any combination, exceeding 2 sq cm (i.e. two, 1x1 cm squares on grid of DFO measuring template) in any one fillet	no single discoloration, nor any combination, exceeding 5 sq cm in any one fillet	any single discoloration, or combination the total surface area of which does not exceed 50% of the total surface area of any one fillet	any single discoloration or combination which exceeds 50% of the total surface of any one fillet
fillet odour	odour characteristic of species	neutral, total absence of odour	slight off-odour, but not objectionable	any objectionable odour

C.I.F.T. 1985

APPENDIX B - GRADING GUIDE

Species:	Date Landed:	Date Inspected:
Name of Vessel:	Name of Fisherman:	Name of Landing Site:
Inspection Location:	Type of Gear:	CFV Number:

Total Catch (lbs): Sample Size (No):

TABLE 1	Fish (No.)											
	Bled											
	Gutted											
	Washed											
	Iced/Temp °F											
	Texture											
	Grade (Table 1)											
TABLE 2	Odour at Neck											
	Odour of Gills											
	General Appearance											
	Eyes											
	Color of Gills											
	TOTAL POINTS											
	Average (Table 2)											
	Grade A (Average <2)											
	Grade B (2.0-2.4)											
	Grade C (2.5-2.8)											
	Grade (Table 2)											
TABLE 3	Blood Clots											
	Texture											
	Discolourations											
	Odour											
	Grade (Table 3)											
	FINAL GRADE											

Grade	Number of Fish	Percent (%)
A		
B		
C		
Reject		

Comments:

SAMPLING SCHEDULE

NUMBER OF FISH IN THE LOT	AVERAGE WEIGHT OF FISH	
	LESS THAN 1 KG NO. OF SAMPLES	1 KG OR MORE NO. OF SAMPLES
100 FISH OR LESS	5	5
101 -130	5	6
131 -160	5	7
161 -190	5	8
191 -220	5	9
221 -250	5	10
251 -300	6	11
301 -350	7	12
351 -400	8	13
401 -450	9	14
451 -500	10	15
501 -600	11	16
601 -700	12	17
701 -800	13	18
801 -900	14	19
901 -1000	15	20
1001-1200	16	21
1201-1400	17	22
1400-1600	18	23
1601-1800	19	24
1801-2000	20	25
2001-2200	21	26
2201-2400	22	27
2401-2600	23	28
2601-2800	24	29
2801-3000	25	30
3001-3200	26	31
3201-3400	27	32
3401-3600	28	33
3601-3800	29	34
3801-4000	30	35
4001-4200	31	36
4201-4400	32	37
4401-4600	33	38
4601-4800	34	39
4801-5000	35	40
EACH 2000 FISH	ADDITIONAL 5	ADDITIONAL 5

Sampling and Sample Size

The sample size, that is the number of fish to be graded, is determined by randomly sampling 10 fish from the lot to determine the average weight of each fish. Divide the average weight into the estimated or actual weight of the lot to be examined to determine the total number of fish in the lot. Based on the total number of fish, the above sampling schedule shall be applied.

1982 QUALITY IMPROVEMENT PILOT PROJECT - GROUNDFISH LANDED QUALITY GRADES

Grades will be assigned using the combination of factors under Texture and Handling Practices (Table 1), Examination in the Round, Gutted or Headed Form (Table 2) and, Examination of Cut Surfaces (Table 3). NOTE: A lot of fish shall be rejected if the percent of reject fish exceeds 10% of the number of fish in the sample, except that a 15% level will apply to fish rejected for reasons of jelliness or chalkiness. Where a lot is rejected, the owner may cull the fish and request a regrading, the results of which are final.

TABLE 1
TEXTURE AND HANDLING PRACTICES

GRADE A:

The fish is firm, resilient and has been properly bled, gutted, washed and iced at sea. Flat fish, except grey sole, shall be bobtailed.

GRADE B:

The fish is reasonably firm, resilient, and gutted, washed and iced at sea. OR

The fish is firm, resilient, and has not been gutted but has been washed and iced at sea.

GRADE C:

The fish is slightly soft and has not been gutted, washed and iced at sea. OR

The fish is moderately soft if gutted, washed and iced at sea.

REJECT: - The fish is:-

1) Tainted, decomposed or unwholesome, OR
2) Rejected by criteria in Table 2 or 3, OR
3) Moderately soft and has not been gutted, washed and iced at sea, OR
4) Soft and has been gutted, washed and iced at sea.

NOTE: Fish shall be properly washed, in clean sea water, to remove excess blood, viscera, slime, and all mud, sand, sea bottom debris and extraneous material.

Fish shall be preserved by the use of finely divided ice sufficient to reduce and hold the temperature at 4°C (40°F) or lower and the ice shall be evenly distributed throughout the catch.

Fish shall be boxed or stored in pens such that the storage height of the fish does not exceed 90 cm (36") and a storage record of the fish catch shall be maintained.

All vessels shall be well maintained (construction, equipment and sanitation) as per FIR.

TABLE 2
EXAMINATION IN THE ROUND, GUTTED OR HEADED FORM

Fish will be graded into Grades A, B, or C by rating all characteristics which are available.

Fish will be rejected if:

a) The flesh at the neck when cut has any sour or putrid odours, OR

b) The odour of the gills is strong, sour or putrid.

GRADE A: - Average Defect Points - Less than 2

GRADES B/C: - Average Defect Points - 2 or more

Characteristics	Defect	Points
Odour at neck when cut	Neutral	2
Gills - Odour	Neutral	1
	Faint-sour	2
	Slight to moderate sour	3
General Appearance	Very light bleaching	1
	Some loss of metallic lustre, some bleaching	2
	Bloom gone and colour faded and bleached	3
Eyes	Slightly sunken or somewhat dull	1
	Dull and or cloudy	2
	More dull, sunken and cloudy	3
Gills	Pinkish - Red to Brownish	1
	Red - some mucous present	2
	Brown, maybe covered with mucous	3

TABLE 3
EXAMINATION OF CUT SURFACES

Texture - Prior to skinning of fillet

A - Firm
B - Reasonably firm
C - Slight to moderate soft
R - Soft or excessive

Odour - Rejected by any objectionable odour

Blood Clots

A - None
B - No combination of blood clots exceeding 4 cm in total maximum dimension in any one fillet.
C - One or any combination of blood clots which exceed 4 cm in total maximum dimension in any one fillet.

Discolouration

A - No single discolouration, nor any combination, exceeding 2 cm in total maximum dimension in any one fillet.
B - No single discolouration, nor any combination, exceeding 5 cm in total maximum dimension in any one fillet.
C - Any single discolouration as combination the total surface area of which does not exceed 50% of the total surface area of any one fillet.

GRADE A:- Jelly: None
Chalky: None or slightly chalky

GRADE B:- Jelly: Slightly jellied
Chalky: Moderately chalky

GRADE C:- Jelly: Moderately jellied
Chalky: Heavy chalky

REJECT: - Any discoloration, the total surface area of which exceeds 50% of the total surface area of any one fillet.

BIBLIOGRAPHY OF GRADING GUIDES AND STANDARDS

1. Grading standard fresh and frozen Atltantic groundfish products DFO-1983-1, Fisheries and Oceans, Fs 38-2/1-1983E.

2. Grading form for the DFO-1983-1 fresh and frozen groundfish product standard, Fisheries and Oceans.

3. Proposed grading standard for fresh and frozen herring fillets, 1982. Fisheries and Oceans.

4. Proposed grading standard for canned snow crabmeat. 1981, fisheries and Oceans Fs 38-2/2-1981E.

5. Handling methods and quality evaluation of fresh Canadian Atlantic Squid (Illex illecebrosus). Ke, P. J., A. D. Woyewoda and M. Fierheller. 1979. Fisheries and Marine Service Technical Report No. 898.

6. Oysters In Fish Inspectors' Procedural Manual 1979. Fisheries and Oceans. pp 5-23 to 5-36.

7. Scallops. In Fish Inspectors' Procedural Manual. 1979. Fisheries and Oceans. pp 5-37 to 5-42.

8. Lobster Meat. In Fish Inspectors' Procedural Manual. 1979. Fisheries and Oceans. pp 5-15 to 5-19.

9. Point of Sale Grading for Atlantic Groundfish - Proposal. 1984. Communications Directorate, Department of Fisheries and Oceans, DFO/1759, I-HQ-84-09E.

APPENDIX C

AIDS TO PHYSICAL GRADING

- INTRODUCTION -

Grading tools to assist in the measuring of color quality and bloodspot size and odour characterization (artificial standard) in groundfish were considered. Investigations were preliminary but did reveal a number of factors regarding grading tools. Many of these parameters are currently under review by Fisheries and Oceans inspection staff.

A. GRADING OF DISCOLORATION IN GROUNDFISH

The need for an improved method of grading for discoloration in groundfish has been identified. Present methods are very subjective and require a certain amount of experience on the part of the grader in order to reach a decision on the severity of the discoloration. Thus, a more objective method of grading would be beneficial.

The consensus of fishery officers, laboratory and plant personnel, and other workers involved in the grading of groundfish was considered essential to the development of these tools and to their eventual acceptance into common use. The considerable practical experience of these people is a most valuable resource in development of these standards.

The recommendation which emerged from conversations with Nova Scotia processors and fishery officers was that a physical color guide, in the form of either color photographs or color chips, was not immediately feasible for the following reasons:

1. natural variation in the flesh color would make it impossible to pinpoint a certain color or even a range of colors that could be classified as "normal" for a particular species;
2. the translucent nature of the flesh makes comparisons with an opaque color chip difficult;
3. discoloration occurs very rarely in cod. In flounder, the pink, yellow, and green discolorations which are occasionally seen are easily identified, and may not necessitate a physical color guide;
4. matching of colors is extremely judgmental, and would become even more so when dealing with faint taints of off-color which would be encountered in fish flesh;
5. a photographic color guide would not be feasible, as the differences which exist are often too slight to be depicted photographically (the only exception to this may be flounder fillets, which sometimes show strong color changes; and
6. The range in quality of photographic reproduction between printers and even between different printing days with the same printer makes a photographic guide impractical.

APPENDIX C - GRADING AIDS

Discussions were held with agricultural researchers involved with color grading of fruits and vegetables. They use color standards for grading of peas, broccoli, etc., but reported that little value was placed on data gathered in this way, because the decisions were too subjective, and because the color standards could not really match the foods.

A color standard for fish must be:

a. representative of fish flesh, even to the novice grader;

b. easily obtained;

c. relatively inexpensive; and

d. highly reproducible (i.e. colors consistent from year to year).

The standard color system used in food evaluation is the Munsell color system which includes a very extensive range of colors (classified by chroma and hue) on glossy color plates, posters, or chips. Each plate has an identification number. However, the expense and unavailability (on a local level at least) of the Munsell system renders it unsuitable for the proposed use. Similar drawbacks were encountered with other less common color systems.

Tool for Discoloration Area Measurement

For blood spot measurement the transparent plastic pocket-sized grading aid introduced by Fisheries and Oceans and in current use seems adequate. Inspection personnel have come to rely on this tool and have confidence in it.

The tool is shown in Figure 1 and includes a grid of squares (each 1 cm per side) for measurement of briuses and blood spots, a square (2.5 cm per side) for fins, and a circle (0.5 cm diameter) for blood clots. Although more descriptors could be printed on its surface to identify the purpose of specific areas, the degree of training required of inspection personnel in all probability renders this extra information unnecessary and distracting.

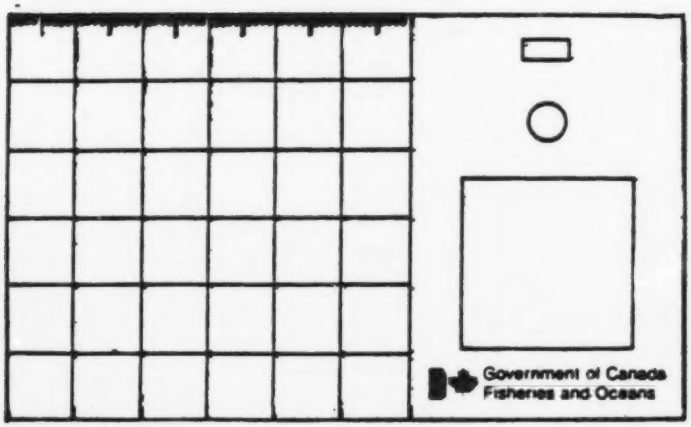


Figure 1. Grading tool utilized by Fisheries and Oceans.

B. AN ODOUR STANDARD FOR GRADING OF COD

LITERATURE REVIEW

Identification of components responsible for the odour of fresh and spoling fish has been the subject of many investigations (Konosu and Yamaguchi). Although ammonia, dimethylamine, and trimethylamine have long been implicated in this role, they may actually have only a modifying effect on the overall odour (Josephson et al., 1983). Recent investigations using various chromatographic techniques have identified volatile components of several fish species. Josephson et al. (1983) determined that the compounds responsible for the odour of whitefish (Coregonus clupeaformis) were (e)-2-nonenal, (E,Z)-2,6-nonadienal, and 6-nonen-1-ol, which imparted the characteristic cucumber-like odour, and (octen-3-ol, 1-octen-3-one, 1,5-octadien-3-ol, 1,5-octadien-3-one, and 2,5-octadien-1-ol, which were responsible for the heavy, plant-like aroma. No sensory tests were conducted to correlate the odours of these compounds with whitefish.

Shiomi et al. (1982) analyzed the volatile compounds of the flat-head (Calliurichthys doryssus) by gas chromatography. They reported that methyl mercaptan and/or dimethyl disulphide were the sulfur compounds responsible for the off-odour of this species.

Lerke and Huck (1977) used gas chromatography to analyze the volatiles from canned tuna. Ethanol was the only peak which increased in magnitude between good, questionable, and decomposed fish.

Rayner et al. (1981) analyzed the volatile components of trout, crab, shrimp, and oysters by gas chromatography and mass spectrometry. Various

compounds were obtained, including, in trout: methanol, methanethiol, ethanol, dimethylsulphide, acetone, and trimethylamine. Similar components were reported for the other seafoods. For headless shrimp, trimethylamine was held responsible for the "strong odour" which characterizes these shellfish held without refrigeration for 24 hours. Indole characterizes the "putrid odour". "Methanol, ethanol, and trimethylamine were excellent indicators of the progressive deterioration that modifies the product odour from very slight to putrid".

Japanese workers have identified the odours emitted from fish meal plants. Volatile fatty acids and volatile sulfur components are responsible for off-odours of press liquid (Makamura et al., 1978). Miwa et al. (1976) reported that trimethylamine, ammonia, dimethylamine, sulfur dioxide, methyl merceptan, hydrosulfide, and volatile fatty acids contributed to odours in fish meal plants.

A study by Miler et al. (1983) noted that hexanal and trimethylamine contribute to the off-odours emitted during the spoilage of fish. They determined the different threshholds for hexanal solutions with and without trimethylamine, and reported that the addition of triemethylamine (at a concentration of $1x10^4$ mM/ml) reduced the odour intensity of the hexanal. No sensory correlation with actual fish odours was made.

McGill et al. (1977) investigated the contribution of hept-cis-4-enal to off-flavours developing during frozen storage of cod. Taste panel assessment of the fish showed good correlation ($r^2=0.84$) of the flavour with this compound. They identified 2,5-dimethylpyrazine as the "animal-like" odour of spoiling cod and various sulfur compounds as the "cabbage smells".

In an extensive study, Wong et al. (1967) identified the volatile compounds found in cod after 0,7, and 14 days of iced storage. Compounds which were identified in cod only after 7 or 14 days were: trimethylamine, propionaldehyde, butyraldehyde, methyl ethyl ketone, methyl propyl ketone, diethyl ketone, and methyl vinyl ketone. Volatiles identified for the first time in spoiling cod were: carbon disulphide, methylene chloride, chloroform, benzene, toluene, methyl propyl ketone, diethyl ketone, and methyl vinyl ketone. No attempt was made to determine the significance of these compounds to decomposition.

Herbert et al. (1975) attempted to isolate and identify "sulphidey" off-odours in spoiling cod. In 10-day old fish, they showed the presence of hydrogen sulfide, methyl mercaptan, and dimethyl sulphide. Subjective evaluation of dilute aqueous solutions of these compounds showed that they produced "sulphidey", "cabbage-like", and "sour sink" odours similar to those produced in spoiling cod.

PREPARATION OF AN ODOR STANDARD

Certain combination of trimethylamine hydrochloride, methyl disulfide, propional, hexanal, Crisco oil and sodium hydroxide solutions where found to somewhat resemble the odor of fresh cod. However from the limited trials a number of observations have arisen:

- odors are not stable and solutions may need to be mixed immediately prior to use;
- a range of odors for fish of various stages of spoilage is required;
- Crisco oil tended to "blend" the odor notes; and
- fish extracts may prove a good starting or base material.

Safety of the standard must also be considered since a liquid form of odor standard could contaminate product during processing. An ideal form of odor reference standard would be impregnated paper or alternately micro encapsulated material on a paper card which could be "activated" when required.

References

1. Herbert, R.A. 1970. Ph.D. Thesis, Aberdeen University, Scotland.

2. Herbert, R.A., Ellis, J.R. and Shewan, J.M. 1975. Isolation and identification of the volatile sulphides produced during chill-storage of North Sea cod (Gadus morhua). J. Sci. Fd. Agric. 26:1187-1194.

3. Josephson, D.B., Lindsay, R.C. and Stuiber, D.A. 1983. Identification of compounds characterizing the aroma of fresh whitefish (Coregonus clupeaformis). J. Agric. Food Chem. 31: 326-330.

4. Konosu, S. and Yamaguchi, K. 1982. The flavor components in fish and shellfish. In Chemistry & Biochemistry of Marine Food Products, (Martin, R.E., Flick, G.J., Hebard, C.E. and Ward, D.R., editors), AVI Publishing, Westport, Conn., p 367-395.

5. Lerke, P.A. and Huck, R.W. 1977. Objective determination of canned tuna quality: identification of ethanol as a potentially useful index. J. Food Sci. 42(3): 755-758.

6. McGill, A.S., Hardy, R. and Gunstone, F.D. 1977. Further analysis of the volatile components of frozen cold stored cod and the influence of these on flavour. J. Sci. Fd. Agric. 28: 200-205.

7. Miler, K.B.M., Kolakowska, A., Czerniejewska-Surma, B., and Sznajdrowska, W.B. 1983. The influence of trimethylamine on the concentration/odour intensity relations in hexanal. Nahrung 27:403-406.

8. Miwa, K., Tokunaga, T. and Iida, H. 1976. Cooking odour and drying odour of fish. Bull. Tokai Reg. Fish. Res. Lab. 86:7-23.

9. Miwa, K., Tokunaga, T. and Iida, H. 1976. Odours in a fish waste meal processing factory. Bull. Tokai Reg. Fish. Lab. 86:1-6.

10. Nakamura, K., Iida, H., Tokunaga, T. and Miwa, K. 1978. Volatile components of fish soluble. Bull. Tokai Reg. Fish. Res. Lab. 93:95-100.

11. Rayner, E.T., Dupuy, H.P., Legendre, M.G., Grodner, R.M., Cook, J.A., Novak, A.F. and Toloday, D.J. 1981. Instrumental analysis of seafood composition. J. Food Sci. 47:76-78.

12. Shiomi, K., Noguchi, A., Yamanaka, H.,, Kikuchi, T. and Iida, H. 1982. Volatile sulfur compounds responsible for an offensive odour of the flat-head, Calliurichthys doryssus. Comp. Biochem. and Physiol. (Part) B 71 B(1):29-31.

13. Wong, N.P., Damico, J.N. and Salwin, H. 1967. Investigation of volatile compounds in cod fish by gas chromatography and mass spectrometry. J. AOAC 50(1):8-15.